EXHIBIT 2:

PUBLICATIONS

DAVID H. PERSING, M.D., PH.D.

PUBLICATIONS

DAVID H. PERSING, M.D., PH.D.

Books:

Persing, D.H., T.F. Smith, T.J. White, and F. Tenover (eds). (1993) Diagnostic Molecular Microbiology: Principles and Applications, ASM Press, Washington, DC

Persing, D.H. (1996) PCR Protocols for Emerging Infectious Diseases, ASM Press, Washington, DC

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Zheng, X., and D.H. Persing. Genetic amplification techniques for the diagnosis of infectious disease. In Rapid Methods. Specter, S. (Ed.)

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Persing, D.H., L. McGinty, C.W. Adams, and R.G. Fowler. (1981) Mutational specificity of the base analogue, 2-aminopurine, in E. coli. Mutation Research 83:25-37.

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JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1209–1215
0095-1137/96/$04.00+0

Vol. 34, No. 5

Sequence Capture-PCR Improves Detection of Mycobacterial DNA in Clinical Specimens

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Received 22 November 1995/Returned for modification 3 January 1996/Accepted 22 February 1996

The rapid identification of mycobacterial DNA in clinical samples by PCR can be useful in the diagnosis of tuberculous infections, but several large studies have found that the sensitivity of this approach is not better than that of culture. In order to improve the sensitivity of detection of mycobacterial DNA in clinical specimens from patients with paucibacillary forms of tuberculosis, we have developed a procedure permitting the specific capture of mycobacterial DNA in crude samples prior to amplification, thereby concentrating the target sequences and removing irrelevant DNA and other potential inhibitors of the amplification reaction (sequence capture-PCR). By using this approach to capture and amplify two different sequences specific for organisms of the *Mycobacterium tuberculosis* complex (IS*6110* and the direct repeat region), it was possible to detect as little as one genome of mycobacterial DNA in samples containing up to 750 μg of total DNA, representing a 10- to 100-fold increase in sensitivity compared with that obtained by purifying total DNA prior to amplification. Detection of the IS*6110* sequence in pleural fluid samples from patients with tuberculous pleurisy by sequence capture-PCR gave positive results in 13 of 17 cases, including 3 of 3 culture-positive samples and 10 of 14 culture-negative samples. In contrast, when total DNA was purified from these samples by adsorption to a silica matrix prior to amplification, only the three culture-positive samples were positive by PCR. The sensitivity of detection of the direct repeat sequence in these samples by sequence capture-PCR was similar to that of IS*6110* and, in addition, permitted immediate typing of the strains from some patients. We conclude that sequence capture-PCR improves the sensitivity of detection of mycobacterial DNA in paucibacillary samples. This approach should be useful in detecting rare target sequences from organisms implicated in other pathologic processes.

Tuberculosis remains a major worldwide health problem and, because of its protean manifestations, must be considered in the differential diagnosis of numerous patients (2, 3, 15). Unfortunately, the standard methods used in the diagnosis of tuberculosis have several important limitations. Microscopic identification of acid-fast mycobacteria is insensitive and, when positive, does not permit identification of the species of mycobacterium identified. Mycobacterial culture may require several weeks to obtain positive results and frequently gives negative results for paucibacillary forms of tuberculosis. These limitations create a variety of problems in the clinical management of patients suspected of having tuberculosis and may lead to delays in initiating appropriate treatment and/or the use of invasive procedures to firmly establish or exclude this diagnosis.

In an effort to overcome these problems, a number of laboratories have evaluated the usefulness of the detection of mycobacterial DNA in clinical samples by techniques based on PCR in the diagnosis of tuberculosis. Several large studies have found that this approach can be used to rapidly diagnose tuberculous infections with a sensitivity that is equivalent to or somewhat less than that of mycobacterial culture (7, 8, 12, 13, 21, 25, 28). Unfortunately, most studies have found that not all samples which are direct examination negative and culture positive are also positive by PCR and that only a minority of culture-negative samples from patients ultimately shown to have tuberculosis are positive by this approach. Thus, in clinical situations in which improvements in diagnostic techniques are most needed (paucibacillary forms of tuberculosis), current PCR techniques have not been of considerable help.

Two obstacles have limited the sensitivity of this approach in the diagnosis of tuberculosis. First, the presence of too much DNA can inhibit PCR, and many clinical specimens (blood, bronchoalveolar lavage fluids, pleural fluids, bone marrow aspirates, tissue biopsies, etc.) contain large numbers of immune and inflammatory cells, a source of large amounts of DNA. Thus, it is necessary to dilute these samples (and consequently the mycobacterial DNA present) prior to amplification. Second, to obtain optimal sensitivity, it is necessary to eliminate inhibitors of the amplification reaction present in clinical samples. Unfortunately, the multistep processes required to obtain highly purified DNA are difficult to apply in routine practice.

To overcome these problems, we have developed an approach that permits the specific capture of mycobacterial DNA in crude samples containing large numbers of human cells, thereby permitting the removal of irrelevant DNA and potential inhibitors present in the original sample prior to amplification. Using this technique, we have demonstrated that this enrichment leads to the anticipated increase in the sensitivity of detection of mycobacterial DNA in standard samples containing known amounts of mycobacterial DNA and in paucibacillary clinical samples from patients with tuberculous pleurisy.

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MATERIALS AND METHODS

Materials. The oligonucleotides used for amplification of a 123-bp fragment of the IS*6110* insertion element (IS1 and IS2) and the direct repeat (DR) region of *M. tuberculosis* (DRa and DRb) have been previously described (11, 16, 23). Oligonucleotides IS3 (13) and DRc (5'-CCCAAAACCCCGAGAGGG) were used for the detection of amplification products by Southern blotting. Capture oligonucleotides for the IS*6110* sequence were Cap-1, 5'-AAAAACGAACG GCTGATGACCAAACTC, and Cap-2, 5'-AAAAAGGAGGTGGCCATCGT GGAAG. These oligonucleotides are complementary to IS*6110* sequences 97 hases upstream of that recognized by IS1 and 39 bases downstream of that recognized by IS2 and therefore do not recognize products amplified by IS1 and IS2. The oligonucleotides were positioned to hybridize with regions devoid of inverted repeat sequences identified by using the STEMLO program. Because the repetitive sequence in the DR region is only 36 bp long, the oligonucleotides used for the capture of DR sequences were identical to the oligonucleotides, DRa and DRb, used to amplify this region, except that 5 adenosine residues were added to the 5' ends. All oligonucleotides were synthesized by Genset (Paris, France). Capture oligonucleotides were synthesized with a biotinylated 5-carbon spacer arm attached to the 5'-end and were purified by high-pressure liquid chromatography. In preliminary experiments evaluating the efficiency of capture of biotinylated oligonucleotides by avidin-coupled magnetic beads, capture oligonucleotides were labelled at their 3' ends with [α-^{32}P]dCTP (Amersham, Slough, United Kingdom) by using terminal deoxytransferase (10).

To evaluate the presence of inhibitory substances in amplification reactions, an internal standard in which the sequences recognized by IS1 and IS2 were added to opposite ends of a 403-bp fragment of plasmid pGEM-3 and which generated a 443-bp fragment when amplified by primers IS1 and IS2 was constructed. Serial dilutions were tested, and the last dilution which gave consistently positive results when amplified in the presence of 0.5 μg of highly purified human DNA (5 μl of a 10^{-8} dilution) was used to verify that specimens could support amplification.

DNA from *M. tuberculosis* H37Rv was purified and quantified by densitometry, and serial dilutions were prepared by using a solution containing 100 μg of human DNA (human placental DNA; Sigma, St. Louis, Mo.) per ml to produce standards containing 0.1 to 100 genomes per 5 μl, assuming a molecular mass of 2.5×10^9 Da for 1 mycobacterial genome (e.g., 1 genome = 3 to 4 fg). To evaluate techniques used for the extraction of mycobacterial DNA, *M. tuberculosis* H37Rv was grown in suspension culture in 7H9 medium, organisms were quantified by limiting-dilution culture, and aliquots containing <10 viable organisms were added to tissues prior to DNA extraction.

Pleural fluid samples. Samples of pleural fluid also submitted for mycobacterial culture were obtained from 17 patients with tuberculous pleurisy evaluated at Hôpital Tenon, Paris, France (age, 38.6 ± 14.5 years; 13 men and 4 women). For 11 patients, the diagnosis was established on the basis of positive culture(s) for *M. tuberculosis* of samples of sputum, pleural fluid, and/or pleural biopsies. For six patients, all mycobacterial cultures were negative and the diagnosis was based on the demonstration of caseating granulomas in pleural biopsies. Cultures of pleural biopsies, performed on seven patients, were positive in four cases. None of the patients had a positive serologic test for human immunodeficiency virus, and none had any other disease known to produce immunosuppression. The volume of pleural fluid obtained from these patients was 5 to 1,000 ml (average, 185 ± 319 ml). Acid-fast staining and mycobacterial culture were performed as previously described (22), except that sputum samples were decontaminated by treatment with 4% sodium hydroxide.

To serve as controls, pleural fluid samples from 25 patients (age, 56.6 ± 15.4 years; 21 men and 4 women) without tuberculosis were also evaluated. The causes of pleural effusion in these patients were as follows: metastatic carcinoma (n = 13), mesothelioma (n = 2), parapneumonic pleural effusion (n = 8), and lymphoma (n = 2). The volume of pleural fluid obtained from these patients ranged from 8 to 1,000 ml (average, 132 ± 253 ml). In seven cases, two different aliquots of pleural fluid were used as control samples.

Solubilization of samples. Pleural fluid samples were centrifuged (2,240 × g; 30 min). Cell pellets or fragments of tissue biopsies were suspended in 500 μl of 100 mM Tris-HCl containing 150 mM NaCl and 50 mM EDTA (pH 7.4), and transferred to 2-ml screw-cap tubes (Eppendorf, Freemont, Calif.) containing 0.5 ml of 0.1-mm-diameter glass microspheres (Biospec Products, Bartlesville, Okla.) and 50 μl of 20 mg of proteinase K (Interchim, Montluçon, France) per ml. Samples were agitated (Mini-BeadBeater; Biospec) for 50 s, allowed to digest overnight at 50°C (Thermomixer; Eppendorf), and agitated again for 50 s, and the supernatant (crude extract) was recovered by centrifugation. Preliminary experiments performed with samples containing small numbers of intact mycobacteria demonstrated that this procedure was highly efficient in releasing mycobacterial DNA.

The DNA in crude extracts was measured by spectrofluorometric assay, as previously described (5). An aliquot containing 5 μg of DNA was removed, and DNA was purified by adsorption to a silica matrix (Geneclean II; BIO 101, Inc., La Jolla, Calif.) as previously described (4, 12). Purified DNA was eluted from the silica matrix into 30 μl of distilled water, and 10-μl aliquots were used for amplification.

Sequence capture. Crude extracts from tissues and cells (final volume, 0.55 ml, containing up to 750 μg of total DNA) were transferred to 1.5-ml Eppendorf tubes, heated at 100°C for 10 min, and cooled to 0°C on ice, and 0.2 ml of 3.75 M NaCl–2.5 pmul each of biotinylated capture oligonucleotides Cap-1 and Cap-2 was added (final volume, 0.75 ml in 1 M NaCl). Tubes were incubated with agitation (Thermomixer) at 60°C for 3 h to allow hybridization. Ten microliters of M-280 Streptavidin Dynabeads (Dynal, Oslo, Nurway), washed according to the manufacturer's instructions, was added, and the incubation was continued for 2 h at 20°C. Magnetic beads were captured (Dynal magnetic-particle concentrator), washed twice with 10 mM Tris-HCl–0.1 mM EDTA (pH 8), and resuspended in water. Two aliquots, each containing 5 μl of beads in 10 μl of water, were used for amplification. Capture of the DR region was performed by analogous techniques, except that the Cap-DRa and Cap-DRb oligonucleotides were used and hybridization performed at 42°C. The procedure is summarized in Fig. 1.

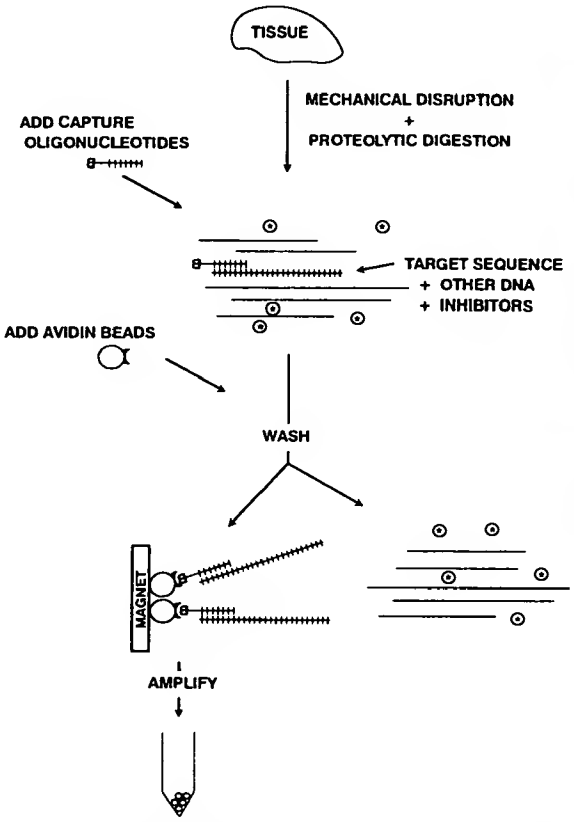

FIG. 1. Schematic representation of sequence capture-PCR. DNA is liberated from tissues or cells, producing a crude extract containing the specific target DNA sequence (hatched bar), human DNA (lines), and potential inhibitors of the amplification reaction (stars). The target sequence is specifically captured by the sequential addition of biotinylated capture oligonucleotides and avidin-coupled magnetic beads. The beads are added directly to the amplification reaction mixture.

Amplification and detection of mycobacterial DNA. Samples for amplification (see above) were suspended in a final volume of 45 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 100 μg of gelatin per ml; 0.2 mM (each) dATP, dGTP, dCTP, and dUTP; 12.5 pmol of each oligonucleotide primer; and 1 U of uracil-*N*-glycolase (Gibco BRL, Gaithersburg, Md.). Samples were incubated at 37°C for 10 min, heated to 95°C for 10 min, and cooled to 80°C in a thermal cycler (Perkin-Elmer, Norwalk, Conn.). Five microliters of a solution containing 1 U of *Taq* DNA polymerase (Appligene, Illkirch, France) was added by using a positive-displacement pipette prior to amplification. For amplification of the IS*6110* insertion element (oligonucleotides IS1 and IS2), the cycling parameters were 95°C for 40 s, 65°C for 40 s, and 72°C for 15 s for 50 cycles. For amplification of the DR region (oligonucleotides DRa and DRb), 2.0 mM $MgCl_2$ was used; the cycling parameters were 95°C for 40 s, 55°C for 40 s, and 72°C for 15 s for 50 cycles. Amplification products were electrophoresed onto agarose gels and transferred to nylon membranes, membranes were hybridized with ^{32}P-labelled oligonucleotides, and positive signals were detected by autoradiography as previously described (23).

Mycobacterial typing. To type mycobacterial DNA amplified in clinical specimens, the spacer oligotyping method described by Kamerbeek et al. (16) was used. Briefly, a 5-μl aliquot of amplification products from positive reactions were reamplified for 25 cycles by using the DR primer set in which the DRa oligonucleutide was biotinylated at the 5' extremity. Aliquots of the amplified

products were hybridized (60°C, 60 min) in a reverse line blotting assay (17) by using a membrane to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *Mycobacterium bovis* BCG had been covalently linked. Membranes were washed at 60°C to remove unbound amplification products and incubated with horseradish peroxidase-labelled streptavidin (Boehringer, Mannheim, Germany), and positive hybridization was revealed by reaction with ECL detection reagents and by exposure of ECL hyperfilm (Amersham, Hertogenbosch, The Netherlands).

Interpretation of results. In experiments evaluating clinical samples, each sample of pleural fluid from a patient with tuberculosis was processed in parallel with four control samples during all steps of the procedure (solubilization of DNA, purification of DNA by sequence capture and adsorption to silica matrix, and amplification). Two types of control specimens, spleen fragments from Wistar rats and pleural fluid samples from patients without tuberculosis, were used. Two identical aliquots of DNA purified by sequence capture or silica matrix adsorption from the same sample were amplified in all cases. Samples were considered positive if one or both of the reactions gave a positive signal on autoradiography. Statistical comparisons were made by using the χ^2 test.

RESULTS

Optimization of PCR. To minimize false-positive results due to carryover of amplified products from prior reactions, all PCRs were performed with dUTP instead of dTTP, and new reaction mixtures were pretreated with uracil-*N*-glycosylase prior to amplification (19). After optimization of reaction conditions, positive results were obtained for amplification of the IS*6110* fragment in 55 of 60 samples containing one genome of DNA from *M. tuberculosis* in 500 ng of human DNA (final volume, 50 μl), 28 of 60 samples containing as little as 0.1 genome, and 0 of 60 samples without mycobacterial DNA. This sensitivity is similar to that we obtained by amplifying this sequence with dTTP (23) and approaches the maximal theoretical sensitivity of the test. (Assuming that *M. tuberculosis* H37Rv contains 15 copies of the IS*6110* sequence and that DNA was fragmented during purification such that each sequence was on a separate fragment, 78 of 100 samples containing 0.1 genomes would contain an amplifiable target.) As previously reported (18), optimal sensitivity was strictly dependent on the total amount of DNA present. When one genome of mycobacterial DNA was added to <1 μg of human DNA, 10 of 10 amplifications were positive, but 3 of 10 and 0 of 5 reactions were positive when the same amount of mycobacterial DNA was amplified in the presence of 2 and 5 μg of human DNA, respectively.

Development of techniques for sequence capture-PCR. Because the presence of excess human DNA impairs the sensitivity of detection of mycobacterial DNA, we developed an approach to selectively purify mycobacterial DNA prior to amplification. Commonly, biotinylated oligonucleotides are attached to avidin-coated beads and subsequently incubated with denatured DNA containing sequences to be captured (direct capture). Positive results can be obtained by this approach for samples containing large amounts of mycobacterial DNA (≥100 genomes). We found, however, that direct capture rarely gave positive results for samples containing 10 or fewer mycobacterial genomes (data not shown), and this technique was abandoned in favor of the two-step capture procedure depicted in Fig. 1.

To ensure that all captured sequences are present in the amplification reaction mixture, it is desirable to directly add magnetic beads containing the captured sequences to the PCR reaction mixture. The addition of up to 5 μl of magnetic beads had no deleterious effect on the amplification of mycobacterial DNA, although larger amounts of beads had progressively prominent inhibitory effects. Thus, capture was performed with 10 μl of beads; beads were subsequently divided into two equal aliquots (5 μl each) prior to amplification. This amount of magnetic beads could completely bind up to 5 pmol of each capture oligonucleotide, but the binding of larger amounts of oligonucleotides was incomplete (Fig. 2). The efficiencies of capture of small amounts of mycobacterial DNA (≤10 genomes) by using 1 and 2.5 pmol each of the two biotinylated capture oligonucleotides were compared and found to be equivalent (data not shown). These results indicate that the use of 2.5 pmol of each oligonucleotide was sufficient to ensure that the concentration of capture oligonucleotides was not a limiting factor in the efficient capture of mycobacterial DNA.

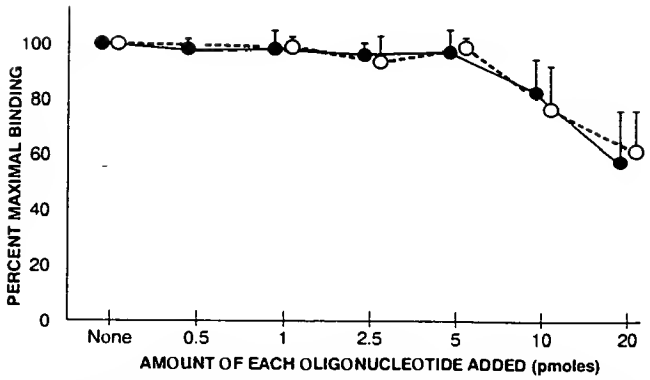

FIG. 2. Binding of capture oligonucleotides by avidin-coupled magnetic beads. Biotinylated capture oligonucleotides were labelled with ^{32}P at their 3′ ends by using terminal transferase, and tracer amounts of radiolabelled oligonucleotide were incubated with 10 μl of avidin-coupled magnetic beads for 2 h at 20°C in the absence (None) or presence of the indicated amounts of each unlabelled capture oligonucleotide, Cap-1 and Cap-2 (solid symbols; $n = 4$) or Cap-DRa and Cap-DRb (open symbols; $n = 3$). Data are the means ± standard deviations of the maximum percentage of oligonucleotide bound, which represented >85% of total radioactivity.

Numerous other factors affecting the efficiency of sequence capture (e.g., solubilization of DNA, composition of the hybridization solution, and times and temperatures during hybridization and binding of oligonucleotides to beads) were also evaluated. To test the overall sensitivity of the conditions defined in these studies, fragments of animal tissues or human immune and inflammatory cells obtained by centrifugation of pleural fluid samples were digested by the established protocol and small amounts of mycobacterial DNA were added to some samples prior to performing capture and subsequent amplification of the IS*6110* sequence. In these studies, 4 of 4 samples containing 100 mycobacterial genomes, 27 of 29 samples containing 10 mycobacterial genomes, and 8 of 13 samples containing 1 mycobacterial genome gave positive results, whereas none of the samples containing no added mycobacterial DNA was positive (Table 1). The positive samples used in these studies contained up to 750 μg of DNA. Thus, it was possible to detect mycobacterial DNA in samples containing as little as 0.001 mycobacterial genome per μg of total DNA, representing a 10- to 100-fold increase in sensitivity over that obtained by amplifying samples without prior enrichment of mycobacterial DNA.

Detection of mycobacterial DNA in pleural fluid samples from patients with tuberculous pleurisy. To determine whether the improved sensitivity of the sequence capture technique would improve the detection of mycobacterial DNA in clinical samples, it was important to use specimens containing only small numbers of mycobacteria. Pleural fluid samples from patients with tuberculous pleurisy were chosen for this purpose. Compatible with the results for prior series (6, 9), mycobacteria were not observed in pleural fluid samples from patients with tuberculous pleurisy by acid-fast staining and only 3 of 17 of these samples were positive by culture (Table 2). For

TABLE 1. Comparison of the sensitivities of sequence capture-PCR in detecting two different mycobacterial sequences, IS*6110* and the DR region[a]

Type of sample	No. of samples positive/no. tested	
	IS*6110*	DR region
Animal tissues or human cells with purified mycobacterial DNA added[b]		
100 genomes	4/4	ND[c]
5–10 genomes	27/29	25/26
1–2 genomes	8/13	2/6
Pleural fluids from patients with tuberculosis[d]	11/15	10/15
Control tissues	0/34	0/25

[a] $P > 0.3$ for all comparisons between IS*6110* and the DR region by Fisher's exact test.
[b] The total DNA was ≤750 μg.
[c] ND, not done.
[d] Only samples for which sequence capture-PCR using both systems was performed.

the detection of mycobacterial DNA by PCR, fluid samples obtained by thoracentesis were centrifuged and DNA was extracted from the cell pellet by mechanical disruption and proteolytic digestion. DNA was purified from an aliquot of the sample by adsorption to a silica matrix, and the remainder of the DNA, up to a limit of 750 μg (total), was used for sequence capture (375 ± 278 μg per sample; $n = 17$).

When total DNA purified by adsorption to a silica matrix was used for amplification of the IS*6110* sequence by the IS1 and IS2 primer pair, only 3 of the 16 samples evaluated were positive; the positive samples corresponded to those that were also positive by culture. To ensure that negative samples could support amplification, an internal standard that generates a 443-bp product when amplified by the IS1 and IS2 oligonucleotides was added to an identical aliquot of each sample prior to amplification. The presence of an amplification product of the expected size was observed in 16 of 16 samples, indicating that the presence of inhibitory substances could not explain the negative results obtained with these samples.

In contrast, when DNA was enriched for mycobacterial DNA by the sequence capture technique prior to amplification of the IS*6110* sequence, positive results were obtained for 13 of 17 samples from patients with tuberculous pleurisy, including the 3 samples that were positive by culture and 10 of the 14 samples that were culture negative ($P < 0.01$; comparing results for DNA purified by sequence capture and adsorption to silica). It is noteworthy that for six of these patients, mycobacteria were never isolated by culture from any specimen submitted. For three of these culture-negative patients, pleural fluid samples gave positive results by sequence capture-PCR; these findings represented the only direct evidence for the presence of *M. tuberculosis* in specimens from these patients.

For each sample from a patient with tuberculosis, three or four control samples were processed in parallel during all steps of the procedure (solubilization of samples, purification of mycobacterial DNA by sequence capture, and amplification). Two fragments of a rat spleen were evaluated to ensure that reagents were not contaminated with mycobacterial DNA and that no transfer of mycobacterial DNA occurred during processing. In addition, one ($n = 2$) or two ($n = 15$) samples of pleural fluid from patients without tuberculosis were tested to evaluate the possibility that mycobacterial DNA could be recovered from individuals without active tuberculosis. None of these control samples gave positive results (0 of 34 animal tissue and 0 of 32 nontuberculous pleural fluid samples).

Amplification of the DR sequence from the *M. tuberculosis* complex by sequence capture-PCR. Sequences present in multiple copies in the *M. tuberculosis* genome are particularly attractive targets for sequence capture. Although most strains of *M. tuberculosis* contain multiple copies of IS*6110*, some strains have few copies; in certain geographical areas, strains not containing IS*6110* are prevalent (27). Therefore, we also developed a sequence capture technique that targets an alternative mycobacterial sequence, the DR sequence. This sequence, which is also specific for the *M. tuberculosis* complex, is present as multiple highly conserved tandem repeats of 36 bp, each separated by a 35- to 41-bp spacer sequence (14). Unlike the DRs, each of these spacers has a unique sequence. Oligonucleotides DRa and DRb, which amplify fragments of variable lengths between two different DR sequences (including the intervening spacer and DR sequences), were used to amplify this region (16).

When samples containing known amounts of purified mycobacterial DNA in 500 ng of human DNA were amplified, positive results were obtained for 11 of 11 samples containing 2 to 10 mycobacterial genomes, 17 of 28 samples containing 1 genome, and 0 of 9 samples containing 0.1 genome. The lower-level sensitivity of the DR system compared with that of the IS*6110* system for the detection of purified mycobacterial DNA is expected. Unlike the IS*6110* sequence, which is dispersed in multiple copies throughout the mycobacterial genome of the mycobacterial strain used as a standard in these studies, the repeated DR sequences are present at a single locus and therefore are likely to be present on a single DNA fragment. Thus, at limiting dilutions (≤1 genome per sample), individual aliquots are less likely to contain fragments with the DR sequence than fragments containing the IS*6110* sequence.

When the sequence capture-PCR protocol was used, however, marked differences in sensitivity between the DR and

TABLE 2. Comparison of the detection of mycobacteria in clinical samples by standard bacteriological techniques and amplification of mycobacterial DNA

Patients[a]	No. of positive sputum samples/no. tested		Result with pleural fluid[b]			
			Bacteriology		Amplification of IS*6110*	
	Acid-fast stain	Culture	Acid-fast stain	Culture	Silica adsorption	Sequence capture
1	0/3	3/3	−	+	+	+
2	0/3	0/3	−	+	+	+
3	0/2	0/2	−	+	+	+
4	0/3	1/3	−	−	−	+
5	0/3	2/3	−	−	−	+
6‡	0/3	0/3	−	−	−	+
7	3/3	3/3	−	−	−	+
8‡	0/3	0/3	−	−	−	+
9	0/3	1/3	−	−	−	+
10	3/3	3/3	−	−	−	+
11‡	0/3	0/3	−	−	−	+
12	0/3	1/3	−	−	−	+
13	0/3	3/3	−	−	ND	+
14‡	0/3	0/3	−	−	−	−
15†	0/3	0/3	−	−	−	−
16‡	0/3	0/3	−	−	−	−
17‡	0/3	0/3	−	−	−	−

[a] †, patient for whom culture of pleural biopsy was positive; ‡, patient for whom all cultures submitted were negative for mycobacteria.
[b] +, positive result; −, negative result; ND, not done.

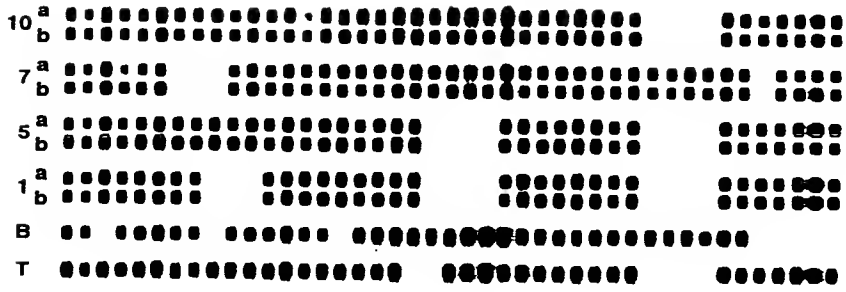

FIG. 3. Typing of mycobacterial strains by spoligotyping after sequence capture-PCR. Sequence capture-PCR targeting the DR region was performed as outlined in Fig. 1 on pleural fluid samples from patients with tuberculous pleurisy. For samples from individuals for which two independent reactions gave positive results, aliquots of the amplification products were reamplified by the DRa-DRb primer pair in which the DRa oligonucleotide was biotinylated. The amplification products were then hybridized to membranes to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *M. bovis* BCG had been covalently linked, and positive hybridization reactions were identified by detecting the presence of biotinylated amplification products using the ECL detection system (Boehringer Mannheim). Spacer oligonucleotides are displayed in numerical order from left to right on the membrane. Shown are the results for four of the five patients (patients 1, 5, 7, 8, and 10) for which the spoligotyping profiles from two independent reactions (a and b) were identical. Note that the profiles are unique for each patient and distinct from those obtained with DNAs from *M. tuberculosis* H37Rv (T) and *M. bovis* BCG (B).

IS*6110* systems were not observed. First, sequence capture-PCR targeting the DR sequence was performed on samples containing small amounts of mycobacterial DNA added to crude extracts of animal tissues or human immune and inflammatory cells containing up to 750 μg of human DNA. Positive results were obtained for 25 of 26 samples containing 10 mycobacterial genomes and 2 of 6 samples containing 1 mycobacterial genome, results not significantly different from those obtained by using the IS*6110* system (Table 1). Similarly, crude extracts of DNA recovered from pleural fluid samples of patients with tuberculous pleurisy, available from 15 patients evaluated by using the IS*6110* system, were also tested by the DR sequence capture technique. Positive results were obtained for 10 of 15 specimens, including all three samples that were culture positive. For 12 samples, the results were concordant between the two systems, although 2 samples positive by using the IS*6110* system were negative by using the DR system and 1 sample positive by using the DR system was negative by using the IS*6110* system.

Typing of mycobacterial strains after sequence capture-PCR. Although all strains of *M. tuberculosis* contain the DR sequence, the spacer sequences present are different for different strains. Kamerbeek et al. (16) have used this observation to develop a technique to type mycobacterial strains on the basis of the hybridization of amplification products of the DR region to a panel of synthetic oligonucleotides specifically recognizing different spacer sequences (spoligotyping). To determine whether the amplification products obtained from the pleural fluid samples of patients with tuberculous pleurisy were adequate to permit rapid typing, this approach was applied to these samples.

It has previously been shown that when extremely small amounts of mycobacterial DNA are used, amplification of only a portion of the DR region may occur, producing incomplete spoligotyping profiles. Although this is not a problem when DNA is extracted from cultured mycobacteria, it is a potential problem when spoligotyping is applied to mycobacterial DNA obtained from paucibacillary clinical samples such as those studied here. To guard against this possibility, typing was restricted to samples for which positive results were obtained for both of the independent amplification reactions and for which the spoligotyping profiles were identical for two independent reactions. These criteria were met for 5 of the 10 pleural fluid samples that were positive for mycobacterial DNA after amplification of the DR region, and the spoligotyping profiles are shown in Fig. 3. In each case, the profiles were distinct and different from that of *M. tuberculosis* H37Rv, the strain used as a positive control in these experiments. Thus, none of the patients was infected with the same mycobacterial strain, and in no case could positive results be explained by the inadvertent contamination of the sample with DNA from another patient or the control strain.

DISCUSSION

In this study, we have developed a new PCR-based strategy, sequence capture-PCR, that permits the rapid enrichment of mycobacterial DNA present in crude extracts of clinical samples prior to amplification and thereby results in a substantial increase in sensitivity of detection of mycobacterial DNA in these specimens. By using samples containing known amounts of DNA, this approach was shown to be 10 to 100 times more sensitive than are procedures in which total DNA is extracted prior to amplification. Furthermore, this improved sensitivity was shown to result in a much higher proportion of positive results when clinical samples from patients with tuberculous pleurisy were tested; only sequence capture-PCR permitted the detection and typing of mycobacteria in a majority of culture-negative specimens from patients with tuberculosis.

The specific capture of nucleic acids by immobilized oligonucleotides has numerous applications in molecular biology but has not found wide application in diagnostic tests. Muir et al. (20) used oligonucleotides coupled to magnetic beads to capture enteroviral RNA prior to reverse transcription-PCR. They found that although this method was simpler to perform, the sensitivity was similar to that obtained by traditional extraction techniques. We found, however, that when oligonucleotides recognizing mycobacterial DNA were directly coupled to beads (direct capture), the efficiency of capture of mycobacterial DNA was much less than that when the biotinylated oligonucleotides were hybridized to mycobacterial DNA in solution and subsequently bound to avidin-coated beads (two-step capture). The reasons that direct capture was less efficient were not studied, but it may result from poor diffusion of the immobilized oligonucleotides and/or steric interference by the large beads. In practice, two-step capture was no more difficult to perform; the only disadvantage is the risk that endogenous biotin could impair efficient binding of biotinylated oligonucleotides. Endogenous biotin was not found in clinical specimens of lungs, lymph nodes, pleural fluids, or

peripheral blood leukocytes. When present (e.g., biopsies of livers and kidneys), it could be removed by pretreating samples with avidin-Sepharose prior to capture (unpublished data).

Our study confirms prior reports (18) that the sensitivity of detection of rare target sequences by PCR is highly dependent on the amount of total DNA in the sample; the sensitivity of detection of mycobacterial DNA was clearly lower in samples containing more than 1 to 2 μg of total DNA in a 100-μl reaction mixture. Because many clinical samples, such as the pleural fluid samples studied here, contain several milligrams of DNA, only a small fraction of the sample can be used when total DNA is studied. In contrast, sequence capture-PCR eliminates essentially all cellular DNA, thereby permitting the analysis of all or the majority of the sample in a single reaction. We have demonstrated that mycobacterial DNA can be detected in a variety of clinical samples, including samples containing large amounts of DNA (e.g., sputum, tissue biopsies, and peripheral blood cells). In addition, sequence capture eliminates potential inhibitory substances present in crude samples. For example, we found that mycobacterial DNA present in tissues containing large amounts of hemoglobin or those extracted with 1% sodium dodecyl sulfate, both strong inhibitors of *Taq* polymerase, could be successfully amplified after sequence capture.

An important finding in the present study was the observation that sequence capture-PCR permitted the detection of mycobacterial DNA in the majority of culture-negative pleural fluid samples from patients with tuberculosis. Prior studies have reported detecting mycobacterial DNA in culture-negative specimens from patients with tuberculosis (7, 11–13, 19, 22, 24), indicating that nonviable organisms can be present in these samples because of the mycobactericidal action of inflammatory cells or loss of viability attendant with sample processing. Nevertheless, in previous studies by us and other groups in which total DNA was amplified, only occasional culture-negative samples gave reproducibly positive results. In contrast, sequence capture-PCR gave positive results for 10 of 14 culture-negative samples. For three of the patients studied here, the detection of mycobacterial DNA by sequence capture-PCR was the only direct evidence for the presence of *M. tuberculosis* in these patients, as multiple sputum, pleural fluid, and pleural biopsy cultures were negative.

Systems permitting the amplification of two different mycobacterial sequences, IS*6110* and the DR region, were developed in these studies. Both were shown to be highly efficient in detecting DNA from as few as 10 mycobacteria in 750 μg of total DNA, and the sensitivities of these two systems for the detection of mycobacterial DNA in tuberculous effusions were not different. These results suggest that sequence capture-PCR can be applied to a variety of different target sequences. Further studies will be needed to rigorously compare the sensitivities of the two systems described here in clinical practice, but two potential advantages of the DR system merit mention. First, the DR sequence is always present in organisms of the *M. tuberculosis* complex in multiple copies; strains not containing this sequence have not been identified. In contrast, the IS*6110* sequence is present in only one or two copies in many *M. tuberculosis* strains and strains lacking IS*6110* have been reported (1, 26, 27). Second, as confirmed in this study, amplification products generated by amplifying the DR region can be used to type the mycobacterial strain detected, thereby permitting rapid identification of community outbreaks or nosocomial infection. Current work in our laboratory is directed at automating the sequence capture-PCR procedure, thereby permitting routine clinical use of this highly sensitive approach.

ACKNOWLEDGMENTS

These studies were supported in part by grants from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés (CNAMTS) and the Fondation pour la Recherche Médicale.

We thank Annelies Bunschoten for excellent technical assistance and Veronique Vincent-Lévy-Frébault for providing the standard mycobacterial DNA.

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CLINICAL MICROBIOLOGY REVIEWS, Apr. 1997, p. 242–256
0893-8512/97/$04.00+0

Vol. 10, No. 2

Relevance of Nucleic Acid Amplification Techniques for Diagnosis of Respiratory Tract Infections in the Clinical Laboratory

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INTRODUCTION

During the last 5 to 7 years, the advantages of diagnostic molecular techniques have been so widely publicized that increasing pressure has been placed on clinical microbiology laboratories to apply them for the detection of a wide variety of infectious agents, especially since test kits for some applications are being made commercially available. In this paper, we review the efficiency and practicability of nucleic acid amplification techniques for the diagnosis of respiratory tract infections.

Before introducing molecular techniques in the diagnostic laboratory, several strategic questions must be addressed: which organisms should be targeted; which clinical specimens should be tested; and do these molecular tests fulfill the required criteria of high sensitivity and specificity, speed, simplicity, and clinical relevance? In general, molecular diagnostic techniques are indicated (i) for the detection of organisms that cannot be grown in vitro or for which current culture techniques are too insensitive, or (ii) for the detection of organisms requiring complex media or cell cultures and/or prolonged incubation times. For respiratory infections, the following organisms meet the criteria described above: rhinoviruses, coronaviruses, hantaviruses, *Bordetella pertussis*, *Legionella* species, *Coxiella burnetii*, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, fungi, and *Pneumocystis carinii*.

This review concentrates on those respiratory agents for which considerable numbers of clinical specimens have been examined. Studies concerning the development of tests for the corresponding pathogens are not considered. Respiratory disease due to cytomegalovirus is not discussed because it does not result from an airborne infection but most frequently from a reactivation of a latent infection in relation to an immunosuppressive state, in which the interpretation of the virological investigations poses particular problems.

The basic principle of any molecular diagnostic test is the detection of a specific nucleic acid sequence by hybridization to a complementary sequence, a probe, followed by detection of the hybrid (21). However, the sensitivity of nucleic acid probe tests that do not involve amplification is lower than that of classical diagnostic tests (191). This lack of sensitivity applies to the detection of respiratory pathogens including rhinoviruses (3, 16), *M. pneumoniae* (71, 102, 103, 176), *C. pneumoniae* (19), and *M. tuberculosis* (150). The main use of the nonamplification probe procedure is in the identification rather than the detection of microorganisms (32, 45).

Thereupon, techniques have been developed to amplify the target nucleic acid or the probe. Any stretch of nucleic acid can be copied by using DNA polymerase, provided that some sequence data are known to allow the design of appropriate primers. DNA replication was made possible in 1958, when Kornberg discovered the DNA polymerase (106). For many years, one of the main applications of this discovery was in the DNA-sequencing procedure of Sanger et al. (166). In 1986, Mullis et al. (132) introduced a reiterative process, PCR, which leads to an exponential increase in the production of the nucleic acid. In view of the immense number of possible appli-

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cations in the most diverse fields, commercial interest was immediately awakened. Alternative nucleic acid amplification techniques were developed and patented, using different enzymes and strategies, but they are all based on reiterative reactions (29, 60, 110, 115, 216).

Nucleic acid amplification techniques can be classified by several criteria. Conceptually, there are those in which the target nucleic acid is amplified and those in which the probe is multiplied (21, 215); from a practical point of view, there are the in-house-developed applications and the commercially available tests. Target nucleic acid amplification techniques include PCR, the strand displacement amplification, and the isothermal RNA self-sustaining sequence replication reaction, from which the commercialized nucleic acid sequence-based amplification (NASBA) and the transcription-mediated amplification (TMA) are derived. The ligase chain reaction (LCR), in the so-called gapped LCR format, is a combination of target and probe amplification. The Qβ replicase amplification (QβRA) involves probe amplification only.

PCR (132) consists of a number of temperature cycles, each cycle consisting of two or three temperature steps: denaturation to ensure the separation of the target DNA duplexes, annealing to allow added synthetic oligonucleotide primers to hybridize to the DNA target, and extension to allow the added DNA polymerase to synthesize complementary DNA strands. In some protocols, annealing and extension occur at the same temperature. After a series of these temperature cycles, the specific PCR product or amplicon, consisting of the two primers bridged by the intervening nucleotide sequence, accumulates. Modifications of the basic procedure are nested PCR (149), multiplex PCR (25), and reverse transcriptase (RT) PCR (149).

In a nested PCR (149), a second round of amplification is performed, using the amplicon of the first round as a target and a pair of primers complementary to sequences within this amplicon, the amplicon of the second reaction being shorter than that of the first. The advantage of nested PCR is increased sensitivity, but this is achieved at the cost of a high risk of cross-contamination, since the tubes containing amplicons have to be opened after the first stage to add new reagents for the second stage. It also increases the specificity of the reaction since the internal primers anneal only if the amplicon has the corresponding, expected, sequence.

In a multiplex PCR (25) several independent amplifications are carried out simultaneously in one tube with a mixture of primers. However, since the annealing temperatures for the respective primer pairs are not necessarily identical, problems of specificity of the individual reactions may result.

In an RT-PCR (149), an RNA target, usually viral RNA, is first transcribed into complementary DNA, enabling the PCR to proceed.

The TMA and NASBA (29, 60) amplify RNA via the simultaneous action of three enzymes: an RT (which also has polymerase activity), an RNase, and an RNA polymerase. The synthesis of cDNA is primed by specially designed oligonucleotide primers, one end of which is a target-specific sequence, while the other end contains a promoter for the RNA polymerase. The RT synthesizes an RNA-DNA hybrid, the RNase digests the RNA component, and the RT synthesizes double-stranded DNA; finally, the RNA polymerase produces numerous RNA copies.

In the LCR (216), after heat denaturation of the double-stranded DNA, two pairs of primers anneal to each strand of the target. A DNA ligase joins the primers, and the ligation product is released by heating and serves as template for new ligations. In the gapped LCR (110), a gap of 1 to 3 bases is left between the primers and is filled in by the action of added DNA polymerase, before the primers are covalently linked by a ligase. In subsequent cycles, the ligated primers act as targets for further annealing and ligation.

In the QβRA (115), a specifically synthesized RNA probe is used. It contains a sequence specific for a target, either DNA or RNA, a sequence to enable the capture of the probe-template hybrids, and a sequence recognized by the Qβ replicase enzyme to start replication. After annealing of the probe to the target, the hybrids are captured, and the probe is removed enzymatically and amplified by the Qβ replicase. This technique is still largely in the developmental stage, the main difficulty being the separation of nonhybridized from hybridized probe before amplification.

Each of the amplification techniques is composed of three parts: sample preparation, amplification, and product detection. The sample preparation step involves primarily the liberation and concentration of the target nucleic acid and the elimination of amplification inhibitors. A great diversity of sample preparation procedures has been described, particularly for PCR. Inhibitors occur frequently and may be difficult to eliminate: heme compounds (79) and polysaccharides in sputum (109), as well as some reagents (67) and components of swabs (207).

The amplification step should aim at maximum sensitivity and specificity through judicious choice of the primers and optimal temperatures when thermocyling is involved, offer maximum protection against contamination, and include proper positive and negative controls. The purpose of the positive control is to monitor the amplification process, particularly to detect inhibitors of the reaction. Concomitant amplification of human β-globin has been used frequently for this purpose. At the same time, it determines the presence of host cell material, which is particularly useful after elaborate sample preparation procedures. However, it requires the introduction of specific primers into the reaction, resulting in a multiplex PCR. To avoid this problem, a PCR for the globin is sometimes performed in a separate tube, but the optimal cycling temperatures for this internal control may differ from those required for the principal reaction. Therefore, specific, positive internal controls are preferred. These are modified amplicons that have been made shorter or longer and are added to each reaction tube. Their ends are identical to those of the target, and therefore they are amplified by the same reagents as the real target, but they are easily differentiated from it by being shorter or longer (6, 38, 44, 64, 96, 105, 137, 148, 151, 167, 188, 197, 198). By adding specific positive internal controls to the samples at the very start of the process, the efficacy of the sample preparation procedure can be assessed. Moreover, the addition of specific internal controls avoids the use of reference organisms or their nucleic acid as positive, external controls, thus eliminating an important possible source of contamination. The addition of a limited amount of internal control should not significantly reduce the sensitivity of the procedure, and it offers greater advantages than disadvantages (198). Internal controls allow also the quantitation of the reaction (96).

Negative controls are target-free samples, usually distilled water, which are subjected to the same manipulations as the test samples. Their purpose is to detect contaminations between reaction tubes. Indeed, after numerous exponential nucleic acid amplifications, there are ample sources of cross-contamination in the laboratory. The greater the number of manipulations, the greater the risk of cross-contamination among the specimens, especially if multiple centrifugations are required. Appropriate measures should be taken to avoid con-

TABLE 1. Diagnostic methods for respiratory viruses

Etiologic agent	Rapid conventional methods available	PCR	
		Relevant	Reference(s)
Adenoviruses	+	No	
Influenza viruses	+	No	27
Parainfluenza viruses	+	No	
RSV	+	No	146
Herpes simplex virus	+	No	
Rhinoviruses	−	Yes	7, 69, 89, 91
Coronaviruses	−	Yes	133
Enteroviruses	−	Yes	91

tamination. These measures include the use of three different rooms with restricted access for each of the reaction steps, the use of appropriate pipette tips, and cleaning of the area by UV irradiation, or the use in the PCR of dUTP instead of dTTP, allowing disintegration of unwanted, possibly contaminating, amplicons by uracil-*N'*-glycosylase (177).

Because of the exquisite sensitivity of nucleic acid amplification tests, there should be a constant awareness of the possibility of false-positive results. These not only are due to cross-contaminations in the laboratory but also may result from contaminations during sampling, particularly when organisms, such as fungi or legionellas present in the environment, are studied. Samples from treated patients may remain positive for prolonged periods (39, 63, 75). For all these reasons, confirmation of the existence of some microorganisms in subclinical infections or a carrier state becomes difficult.

In the PCR and the LCR, the amplicons can be detected by gel electrophoresis, followed or not by solid- or liquid-phase hybridization with a specific probe, by fluorescence (88), or by an enzyme immunoassay (EIA) reaction. Hybridization can increase the sensitivity of the detection 10- or 100-fold. The amplicons of NASBA and TMA are detected by hybridization or by a commercial luminescence reaction (41), and those of the QβRA can be detected by an incorporated fluorescent dye.

At present, PCR is undoubtedly the most widely used amplification technique, probably because it was the first one described and was introduced rapidly in innumerable laboratories for a wide variety of applications. Commercial formats of PCR (Roche), TMA (MTDT, GenProbe), NASBA (Organon Teknika), and LCR (Abbott) have been developed, particularly for infectious agents for which large numbers of clinical specimens are tested: sexually transmitted agents (*Neisseria gonorrhoeae*, *C. trachomatis*, human immunodeficiency viruses), hepatitis C virus, and *M. tuberculosis*. In these formats, the amplicon is detected either by a semiautomated EIA reaction (Roche) or by an electrochemiluminescence procedure or a hybridization reaction (Organon Teknika), or it is coupled to an existing acridinium ester luminescent nucleic acid probe technique (GenProbe) or a previously developed, automated EIA technique (Abbott).

In-house tests are more versatile and can easily be applied to any target by switching to the appropriate primers and, if necessary, adapting the cycling temperatures accordingly.

MOLECULAR DIAGNOSTIC TECHNIQUES FOR ACUTE RESPIRATORY TRACT INFECTIONS

Viruses

Table 1 illustrates the present situation for the diagnosis of adenovirus, influenza virus, parainfluenza virus, and respiratory syncytial virus (RSV) infections for which rapid conventional techniques are available: influenza virus and RSV can be detected in the clinical specimens by immunofluorescence and parainfluenza virus and adenovirus can be detected by immunofluorescence after incubation for 48 h in shell vial cultures (147). In these cases, nucleic acid amplification techniques have no added value in terms of sensitivity or rapidity. In one study (27), comparing PCR with conventional techniques for the detection of influenza virus, the authors concluded that there are no arguments for the introduction of PCR for the diagnosis of influenza virus infection. In a study by Paton et al. (146), PCR for RSV had a sensitivity of 94.6% and a specificity of 97%; the molecular technique detected 1% of cases undiagnosed by culture and EIA. Clearly, PCR does not represent significant improvement over existing methods for the detection of these viruses.

Rhinoviruses and coronaviruses grow poorly in cell culture. In addition, rapid immunofluorescence and/or culture techniques are not available for the direct detection of these viruses in clinical specimens (7, 69). Typically, rhinoviruses are isolated in roller cultures, sometimes after several blind passages, followed by acid lability testing. More than 100 serotypes are known. PCR is much more sensitive than is culture (136): Ireland et al. (89) and Johnston et al. (91) detected five and three times as many rhinoviruses by PCR, respectively, compared with the best available cell culture techniques. In another study (59), significantly more multiple-virus infections by RSV, parainfluenza viruses, and rhinoviruses were detected by RT-PCR than by culture. However, some technical details must still be worked out. To detect the large number of rhinovirus serotypes, regions within the conserved noncoding 5′ untranslated region of the genome are amplified (54), leading to cross-reactions with many enteroviruses. Several methods have been used to detect rhinoviruses specifically: a nested procedure, the use of primers spanning a region between the 5′ untranslated region and the VP2/VP4 region, hybridization with specific probes (69), and differentiation on the basis of the size of the amplicons (89, 141, 196) or sequencing (131). Nevertheless, Johnston et al. (91) could identify only 8 of 30 positive samples as rhinoviruses on the basis of either acid lability or the length of the amplicon, with 73% remaining "unclassified picornaviruses." Another problem emerging from studies on human rhinoviruses by PCR is whether healthy carriers exist: 12 and 4% of samples from asymptomatic children and adults, respectively, were positive for picornavirus by PCR (91).

Clearly, there is still more to learn about the epidemiology of rhinoviruses, particularly in children, infants, and the elderly. Molecular diagnostic techniques offer the necessary tools.

A PCR based on the genomic sequences of the two known human coronavirus strains, 229E and OC43, is available (133), and it is highly likely that more, as yet uncultivated, human coronaviruses remain to be detected. No extensive studies to define better the role of coronaviruses in respiratory infections have been undertaken.

Hantavirus pulmonary syndrome, a rodent-borne infection, appeared in 1993 and 1994 in the New Mexico-Arizona-Colorado area. It is characterized by fever, myalgias, headache, and cough, followed rapidly by respiratory failure. Antibodies against heterologous hantavirus antigens were initially used to identify the causative agent, and then the hantavirus genome was detected by PCR in autopsy specimens (135). Specific genetic recombinant-derived proteins were prepared from viral genomic sequences amplified from tissues obtained from patients who died of confirmed hantavirus illness (108). Since the virus has not yet been cultured, PCR with specific primers and serology are the only diagnostic possibilities.

Rapid diagnostic techniques for respiratory pathogens are not only important for clinical-epidemiological reasons but are also useful so that treatment can be appropriately initiated within the first 24 h or halted when the symptoms are found to be caused by another microbial agent.

Bacteria

Bordetella pertussis. Despite the routine immunization of children, pertussis continues to be an important disease in infants and young children. During the last 2 years, there has been a resurgence of pertussis in the United States (24), Italy, the Russian Federation, and Sweden (165). In 1994, approximately 3,500 to 4,000 cases were reported to the Centers for Disease Control and Prevention in the United States (24). These figures probably underestimate the true incidence of pertussis because of the difficulty in confirming the diagnosis (70, 182). The major reservoir for pertussis now appears to be previously vaccinated adolescents and adults with atypical and often unrecognized symptoms of pertussis. Making the clinical diagnosis of pertussis in this reservoir is more challenging because many of these patients do not have the classic coughing paroxysms or "whoops."

The conventional laboratory diagnosis of pertussis has relied on culture, direct immunofluorescence, and serologic testing. Each of these methods has problems with either sensitivity or specificity (47, 70, 182). Diagnosis by culture is specific but not very sensitive since most individuals are culture negative at the time when clinical symptoms are apparent. Direct immunofluorescence is prone to a large number of false-positive results, and when used on a single specimen, serologic testing is often nonspecific. Follow-up confirmation with a second specimen would result in a 3- to 4-week delay in the diagnosis. These problems have led to an inability to confirm the diagnosis in many patients, and therefore nucleic acid amplification techniques, in practice PCR, have been used (8, 40, 47, 68, 72, 73, 169, 170, 199). The presence of a repetitive gene element in *B. pertussis* increases the sensitivity of the PCR. The reaction allows also a clear-cut distinction between the pathogenic *B. pertussis* and the usually nonpathogenic *B. parapertussis* (199).

An unexpected origin of false-positive PCR results for *B. pertussis* was described by Taranger et al. (187). Pharyngeal samples were obtained in a room that was grossly contaminated with pertussis DNA because killed, whole-cell pertussis vaccine was administered in the same room.

In a recent report (123), several aspects of PCR-based detection of *B. pertussis* were discussed. The main conclusions, which we can support, were that (i) there are no comparative studies between the different PCR procedures; (ii) although the PCR procedures used in different laboratories can detect 80 to 100% of the culture-positive samples, the percentage of PCR-positive samples that were culture negative differed by 13 to 88%; (iii) there is need for rigorous control of false-positive and false-negative results; (iv) questionable results must be confirmed by a second method; and (v) PCR-positive results are acceptable only for individuals with classical symptoms of pertussis. The clinical and epidemiological significance of a PCR-positive result in someone with mild or no symptoms should be interpreted with caution, and, if possible, other markers, such as serologic tests or epidemiologic data should be used in addition. Finally, it is too early to recommend a standard PCR technique for the detection of *B. pertussis* in clinical specimens, because no comparative studies have been done.

***Legionella* species**. Legionellae are ubiquitously distributed in natural and man-made water systems (49, 206). Respiratory infections caused by *Legionella* spp. often occur in immunodeficient persons. Cultures of bronchoalveolar lavage specimens take a minimum of 48 to 72 h to grow, and plates should be incubated for 7 days. Jaulhac et al. (90) applied PCR retrospectively to frozen bronchoalveolar lavage specimens. They confirmed all culture-positive specimens and found additional specimens positive by PCR from patients whose clinical features were in accordance with legionellosis. Kessler et al. (98), in a prospective study combining a rapid DNA extraction procedure with a commercial kit for the amplification and detection of legionellae in environmental samples, detected the organisms in all specimens later confirmed by culture. In another study (125), legionellae were detected by PCR but not by conventional culture.

In an effort to detect *Legionella* infections by the examination of specimens obtained by less invasive procedures, Maiwald et al. (120) examined urine specimens from experimentally infected guinea pigs and patients by an EIA and by PCR. PCR was more sensitive than EIA in detecting legionellae, and two urine samples were intermittently positive, indicating that DNA is not continuously excreted. The advantage of PCR over EIA is that PCR is a genus-specific reaction whereas antigen detection must be performed with a variety of serogroup reagents to cover the spectrum of possible causative species. The authors concluded that a more detailed prospective study of hospitalized patients with pneumonia is warranted. Their results also illustrate the recurring problem of contamination associated with amplification techniques, since 3 of 30 control samples from patients with urinary tract infections were positive, possibly as a result of contamination by hospital water.

The need for nucleic acid amplification techniques for *Legionella* infections can be questioned in view of their relatively easy isolation from respiratory specimens within a moderate time span and the ability to prevent nosocomial legionellosis by control of legionellae in the hospital plumbing system (114). PCR may be more suitable for the detection of legionellae in environmental specimens to avoid overgrowth by contaminating organisms (119).

Coxiella burnetii. *C. burnetii* is a fastidious intracellular bacterium. Different strains show heterogeneity in their growth conditions, with some being very difficult to culture in vitro. The isolation of *C. burnetii* was greatly improved and facilitated by application of the shell vial assay technique (159), which produced results within 6 days. A PCR for *C. burnetii* (181) has been shown to be very sensitive and specific and is able to produce results within 6 h. It can be applied to inoculated shell vials or directly to clinical specimens. For the time being, this procedure will remain restricted to reference laboratories in countries or areas where the disease does occur, as illustrated recently by To et al. (194).

***Chlamydia* species**. Three *Chlamydia* species are responsible for human respiratory infections: *C. psittaci* and *C. pneumoniae* in adults and older children, and *C. trachomatis* in newborns, who are infected during delivery.

The last organism has been implicated, by serology (2), in 3 to 18% of all cases of infant pneumonitis. Although nucleic acid amplification techniques for the detection of *C. trachomatis* in genitourinary specimens have been intensively studied, there are no such studies on respiratory specimens. It could well be that the techniques used for genitourinary specimens cannot be applied unchanged to respiratory specimens, particularly the specimen preparation procedure (41).

C. psittaci may be an important human pathogen in some areas and may be underdiagnosed on the basis of serologic testing alone. Since respiratory infections by *C. trachomatis* and *C. psittaci* occur sporadically, there has been less need or

opportunity for the application of amplification techniques for these infections. Several research groups have developed a two-step procedure for the successive detection of organisms belonging to this genus and their subsequent identification to the species level, by the amplification of a common genus-specific DNA sequence followed by digestion with restriction enzymes (80, 160, 210) or by a nested PCR (195). None of these procedures has been applied on a significant scale.

The role of *C. pneumoniae* in disease was discovered relatively recently, but the insensitivity of cell culture techniques has hampered extensive clinical and epidemiological investigations. In addition, serologic tests are labor-intensive, since they rely on microimmunofluorescence tests for detection of both immunoglobulin M (IgM) and IgG. Serologic investigations seem to indicate that the culture technique fails to detect many infections. However, taking into account the shortcomings of serologic testing, in terms of specificity and sensitivity (58), it can be surmised that the techniques available fail to diagnose *C. pneumoniae* infections to an unknown extent, although the organism does not seem to be a common cause of respiratory infection in children (65). Therefore, several PCR primer sets have been developed to detect either outer membrane or 16S rRNA coding genes (10, 19, 55, 58, 66, 143, 157, 160).

One of the difficulties in evaluating nucleic acid amplification tests for the diagnosis of *C. pneumoniae* infections is the choice of the reference or "gold standard." Because culture is relatively insensitive, many studies refer to serologic results, considering the presence of IgM, a fourfold increase in antibody titers during and after the acute disease episode, or an IgG titer of at least 512 to be significant. The presence of clinical symptoms cannot be taken into account, since asymptomatic infections by *C. pneumoniae* have been documented by culture and PCR (84).

In addition to this problem of the appropriate reference method to use for the detection of *C. pneumoniae*, inhibitors of PCR are common components of the specimens. Some solutions have been proposed, including the use of samples such as gargled water, throat swabs, or nasopharyngeal swabs instead of nasopharyngeal aspirates or sputum (157, 195), alternative sample treatment methods (62, 117), and introduction of a nested PCR (11).

In all studies in which they were compared, PCR detected 10 to 20% more cases than culture, but in turn serologic determination detected 10 to 20% more cases than PCR. In one study (58), when compared with the combination of a positive culture and direct immunofluorescence test, the PCR had a sensitivity of 76.5% and a specificity of 99%; when compared with the combination of a positive PCR and direct immunofluorescence test, the sensitivity of culture was 87.5%. In the same study, only 8 acute-phase serum specimens (23%) of the 35 *C. pneumoniae* culture- or PCR-positive patients had a diagnostic antibody titer, as did 18.8% of those from 80 asymptomatic persons. Thom et al. (192) diagnosed 21 cases by serologic testing among 743 middle-aged and older patients; 15 of the patients were positive by PCR. Gaydos et al. (56) studied 132 *C. pneumoniae* culture-negative BAL specimens from 108 immunocompromised patients. A total of 20 *C. pneumoniae* infections were diagnosed: 8 by PCR, 4 by PCR and serologic testing, and 8 by serologic testing alone. In this study, PCR and serologic testing had a sensitivity and specificity of 33.3 and 91%, respectively, and both detected 60% of the cases. Thus, it seems that both conventional culture and PCR diagnose only a fraction of the total number of cases and that the diagnosis of individual infections by serology is by no means straightforward, due to the occurrence of many false-negative and false-positive results.

Many aspects of the diagnosis of *C. pneumoniae* infections by amplification techniques remain to be explored. There is need for an internal control; for comparisons of different types of samples, sample preparation methods, and primers; and for several amplification techniques to be performed on the same specimens.

Mycoplasma pneumoniae. *M. pneumoniae* grows slowly in vitro, requiring 2 to 4 weeks for colonies to appear. Therefore, research laboratories have identified several genomic sequences suitable for amplification, including the P1 gene (87), the 16S rRNA gene (201), and a species-specific protein gene (116). In clinical studies, the sensitivity and specificity of amplifications based on these sequences were 90 to 94% and 97 to 100%, respectively (34, 57, 86, 94, 112, 116, 122, 176, 178, 193, 201). PCR also detected *M. pneumoniae* in specimens from 1 to 3% of healthy subjects (116, 193) or convalescent patients, raising the possibility of a carrier state or persistence of the organism in the respiratory tree. In a recent study (86), 371 nasopharyngeal aspirates from children with acute respiratory infections were examined for viruses by rapid conventional techniques and for the presence of *M. pneumoniae* by culture and several different PCR protocols in two laboratories. Each laboratory applied one sample preparation method: freezing-boiling or isothiocyanate treatment, followed by phenol-chloroform extraction. Prepared samples were exchanged between laboratories. In both laboratories, identical primers were used in the PCR directed against the P1 gene, while one laboratory also used primers against the 16S rRNA gene. A specific internal control for the P1 amplification was included (198). Samples were defined as positive if (i) culture was positive for *M. pneumoniae*, (ii) culture and PCR for the P1 and/or the 16S rRNA genes were positive, or (iii) PCR was positive for both the P1 and 16S genes after a particular extraction procedure. Samples positive by PCR for only one of the primer pairs were considered as contaminants. Compared with PCR, culture had a sensitivity of 61%. For the PCR, depending on the preparation method used, sensitivity with the P1 primers was 76.9 to 92.3% on inspection of the electrophoresis gel and 92.3% after hybridization. The specificity was 100%. Depending on the sample preparation method, amplification of the 16S rRNA gene had a sensitivity of 53.8 to 84.6% on visual inspection of the electrophoresis gel and 69.2 to 92.3% after hybridization. The specificity was 100%. It was concluded that, provided a specific positive internal control is used, sample preparation by freezing-boiling combined with PCR for the P1 gene and amplicon detection by visual inspection of the electrophoresis gel could be recommended for clinical use, although the best results were obtained by hybridization with a labeled probe. False-positive results occurred in 0.2% of the reactions. It remains to be seen whether the finding of Resnikov et al. (163) that throat swabs contain significantly fewer PCR inhibitors than do nasopharyngeal aspirates is confirmed and that the effect does not simply result from dilution.

In the same study by Ieven et al. (86), *M. pneumoniae* was found in 3.5% of the samples but significantly more often (6.9%) in those from children older than 2 years of age. *M. pneumoniae* was the third most common etiologic agent of acute respiratory infections in children, after RSV and influenza virus. In lower respiratory infections, such as bronchopneumonia and pneumonia, *M. pneumoniae* was found as frequently as RSV. PCR is unquestionably an important step forward for the diagnosis of *M. pneumoniae* infections.

Mycobacterium tuberculosis. Amplification techniques for the diagnosis of tuberculosis have attracted considerable interest, particularly with the hope of shortening the time required to detect and identify *M. tuberculosis* in respiratory specimens

TABLE 2. Evaluation of PCR for *M. tuberculosis* in different studies

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Abe et al. (1)	135	28	81.3	84.2	94.2	100	81.3	84
Beige et al. (9)	103	47	98		70		75	
Clarridge et al. (28)	>5,000	4.4	83.6	86.1	98.7	100	94.2	98.4
Forbes and Hicks (51)	734	11		85.2		97.7		83.3
Kocagöz et al. (104)	78	49		87		96		97
Miller et al. (126)	750	21	78.2	92.3				100
Miyazaki et al. (129)	323	13	97		92	100	82	100
Nolte et al. (137)	313	40	91		100		100	
Shawar et al. (175)	384	18	74	80	95	97	77	86
Yuen et al. (218)	519	8	96		85	100		

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.

such as sputum or BAL samples. It is in this field of clinical microbiology that most amplification procedures, developed both in-house and in commercialized formats, have been evaluated.

(i) Technical aspects. Many different DNA amplification targets have been proposed, such as genes encoding the 32-kDa (179), the 38-kDa (129, 219), and the 65-kDa (145, 152) antigens and the *dnaJ* (183, 184), *groEl*, and *mtb-4* genes (104, 220). Some of these are genus or group specific, with species identification requiring subsequent restriction enzyme treatment or hybridization. The target most frequently amplified is the IS*986* or IS*6110* repetitive element (43, 77), which is present at 10 to 16 copies in most *M. tuberculosis* complex isolates, thereby increasing the sensitivity of the amplification reaction. In comparative studies, tests with the IS*6110* primers were generally more sensitive and more specific than those with IS*986* (37, 76, 208). Recently, however, *M. tuberculosis* isolates without this insertion element have been discovered in Southeast Asia (33, 202, 219).

Numerous techniques for sample preparation have been proposed, including boiling; freezing-boiling; shaking with glass beads (100); sonication (17); chloroform (213), proteinase K or "chelex" (36) treatments and combinations of these treatments; resin treatment (4); and more complex nucleic acid extraction methods (14). The commercial kits furnish their own sample treatment reagent.

Some PCRs are performed with dUTP instead of dTTP, allowing decontamination with uracil-*N'*-glycosylase (217). Both single and nested PCR formats (129, 152, 176, 213) have been applied, sometimes with the explicit purpose of overcoming PCR inhibitors.

Internal controls have been used (6, 38, 44, 105, 137, 139). However, they were only occasionally added to the specimens before the DNA extraction procedure, as was done by Kolk et al. (105). By being present during the entire procedure, an internal control not only detects inhibitors but also monitors the efficacy of the sample preparation method. Inhibitors have been detected in 3.7 to 16% of clinical samples (28, 51, 139). Curiously, Nolte et al. (137) detected inhibitors in 17% of the samples with β-globin primers but only in 10% with a specific internal control.

(ii) Results on sputum specimens with in-house PCR tests. Table 2 presents the results of nine studies in which IS*6110* was used as the amplification target. Some of these studies were done on a series of specimens with a high prevalence of positive samples. It should be remembered that for a constant rate of false-positive tests, the positive predictive value of a test decreases drastically when the prevalence of infection is low, as is the case in industrialized countries. In a population with a prevalence of <5% (in most Western European countries [139], the prevalence of positive samples is 3 to 4%), false-positive rates of 1 to 5% can lead to overdiagnosis of 50% or more of cases.

In general, the authors of the studies present their results first as "crude results", i.e., as produced by the test and thereafter as "revised results," i.e., after considering the discrepancies between the test results and the corresponding clinical information. Some authors include culture-negative, clinically diagnosed cases of tuberculosis among the "true-positives," sometimes even based on favorable response to anti-tuberculosis treatment, and thereby increase the specificity and positive predictive value of the test. None of them formulated a standard definition of a positive case except for Noordhoek et al. (139), who used the following definition of a true-positive specimen: (i) *M. tuberculosis* was cultured; or (ii) direct microscopy and PCR were positive but culture was negative; or (iii) direct microscopy and culture were negative but PCR was positive and other material from the patient was positive on culture or had been positive in the past.

None of the published studies observed a statistically significant difference between culture and the amplification technique (99). However, sensitivity and specificity are calculated as a function of the culture technique, since this is the reference method used in the absence of a better definition of a positive case of tuberculosis. In the studies, specificities vary between 85 and 100% but sensitivities are usually lower, between 74 and 97%. In one study on over 5,000 specimens (28) with a 4.4% prevalence of positive results, sensitivity, specificity, and positive predictive values were 84, 99, and 94%, respectively. By applying two primer systems in a multiplex PCR, Beige et al. (9) attained a sensitivity of 98% but a specificity of only 70%.

However, the main criticism of the use of PCR for the diagnosis of tuberculosis is a result of the separate analyses of the sensitivities of smear-positive and smear-negative, culture-positive specimens in different studies (Table 3). The test sensitivity in smear-positive cases is 88 to 100% but drops to between 50 and 92% in smear-negative cases.

One of the reasons for the lack of sensitivity may be the sample preparation method. Except for one study (139), all the procedures were applied to homogenized and decontaminated specimens as used for culture. Although this may seem appropriate when amplification techniques are compared with culture, it is not logical and may not even be optimal. In all studies

TABLE 3. Results of PCR for *M. tuberculosis* for smear-positive and smear-negative specimens

Study (reference)	PCR sensitivity (%) in different studies		
	Overall	Smear and culture positive	Smear negative, culture positive
Abe et al. (1)	84	96	50
Clarridge et al. (28)	86	94	62
Forbes and Hicks (51)	85	88	71
Miller et al. (126)	92	98	78
Nolte et al. (137)	91	95	57
Shawar et al. (175)	74	90	53
Yuen et al. (218)	96	100	92

of diagnostic amplification techniques for microorganisms other than *M. tuberculosis*, samples are divided before being allocated to the reference and amplification techniques and are thereafter prepared separately as required for each. If this were done for tuberculosis, half of the original specimen would be lysed and the nucleic acid target would be solubilized, concentrated, and introduced into the amplification reaction, thus possibly maximizing the sensitivity. In the case of paucibacillary specimens, there is a delicate balance between amplification procedures and culture. Compared with the amplification procedures, a significantly greater volume of specimen is introduced into the culture media, thus favoring the latter. However, the decontamination procedures kill 70 to 90% of the viable bacilli in the inoculum (107, 217), favoring the alternative approach. This aspect of sample preparation has been studied by Goessens et al. for the detection of *C. trachomatis* in genital specimens (63) and merits investigation for tuberculosis.

Only Noordhoek et al. (139) divided the specimens into two portions, one for conventional detection methods and one for PCR, directed at the IS*6110* element. Unfortunately, their analysis was done with a mixture of respiratory and nonrespiratory specimens, including pleural fluid, urine, and biopsy specimens. The sensitivity and specificity were 92.1 and 99.8%, respectively. PCR was negative for nine smear- and culture-positive samples. The corresponding isolates were tested and did contain the IS*6110* fragment. The authors ascribe these failures to an unequal distribution of a small number of mycobacteria present in the samples, since in each of these cases, only one or two of the three Loewenstein-Jensen culture tubes that were inoculated in parallel were positive. In this study, amplification of DNA extracted from half of the sputum specimen was not superior to culture of the other half.

In this connection, the sequence capture procedure recently described by Magiapan et al. (118) for pleural fluid specimens could be a significant advance. In this procedure, biotinylated oligonucleotides hybridize with mycobacterial DNA in the specimen and are subsequently bound to avidin-coated beads, which are introduced into the PCR mixture. Of 17 samples 13, including 3 of 3 culture-positive samples and 10 of 14 culture-negative samples, gave positive PCR results. Results of the application of this procedure to sputum specimens are eagerly awaited. The use of more appropriate primers could also enhance the sensitivity of the reaction, since even for a particular DNA sequence, different primers may result in different test sensitivities (74, 220).

Efforts to increase the sensitivity by performing a PCR on 25 μl instead of 5 μl of specimen were hampered by an unacceptable increase in the level of inhibitors (6). In contrast, by increasing the sample volume in the commercially available TMA (GenProbe MTDT) from 50 to 500 μl, one group (13) increased the sensitivity from 71.4% (obtained in a previous study [12]) to 83.3% without a loss of specificity (13).

The effectiveness of PCR for tuberculosis is related to the experience and accuracy of the personnel conducting the assay. This was illustrated by an external quality control study of seven laboratories which were tested with sputum samples spiked or not spiked with *M. tuberculosis* BCG (138). Each laboratory used its own protocol for specimen treatment and amplicon detection, but in each case the amplification target was IS*6110*. In general, false-positive rates varied between 0 and 20%, but the rate in one laboratory reached 77%; sensitivities varied between 2 and 90%. A second external quality control study of 30 laboratories, organized more recently by the same authors (140), showed no improvement: 56% of participants produced false-positive results in 5 to >50% of the samples.

(iii) Results on sputum specimens with commercially available amplification tests. The commercially available PCR (Amplicor; Roche) and TMA (Mycobacterium Tuberculosis Direct Test [MTDT]; GenProbe) test give results comparable to those obtained with in-house PCR tests (Tables 4 and 5). Sensitivities vary between 70 and 100%. The results of the MTDT for smear-positive and smear-negative specimens, respectively (Table 6), are comparable to those obtained by PCR.

Schirm et al. (168) compared an in-house PCR and the commercial PCR (Amplicor) on 504 specimens. The sensitivity of the in-house test, 92.6%, was superior to that of the Amplicor system, 70.4%, although the specificity was identical for both. More samples were inhibitory in the commercial test

TABLE 4. Evaluation of the commercially available PCR (Amplicor) for *M. tuberculosis*

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Carpentier et al. (20)	2,073	9	86		98		94.5	
D'Amato et al. (31)	985			66.7		99.7		91.7
Gleason et al. (61)	532		95		96			
Ichiyama et al. (85)	422	29	97.8		96	98.7		
Moore and Curry (130)	1,009	16	83	87	97	100		
Schirm et al. (168)	504	6	70.4		98			
Vuorinen et al. (205)	256		84.6	82.8	99.1	100		100
Wobeser et al. (214)	1,480	9.5		79		99		93

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.

TABLE 5. Evaluation of MTDT for the detection of *M. tuberculosis*

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Abe et al. (1)	135	28	90.6	91.9	95.1	100	85.3	100
Bodmer et al. (12)	617	3	71.4		99		71.4	
Ichiyama et al. (85)	422	29	100		90.1	99.3		
Jonas et al. (92)	758	16	79.8	82.4	96.7	99.4	82	93.8
Miller et al. (126)	750	19	83.9	91	95.3	98.5	82	97
Pfyffer et al. (154)	938	8	92.9	93.9	96.2	97.6	68.4	94
Portaels et al. (156)	497[d]	4	86		96		50	80.7
	418[e]	71	97		69		89	
Vlaspolder et al. (203)	412	14	96.7	98.4	97.7	98.9	88.1	93.8
Vuorinen et al. (205)	256	13	84.6	86.2	98.7	100	100	

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.
[d] Belgian population.
[e] African population.

than in the in-house version. Both Ichiyama et al. (85) and Vuorinen et al. (205) compared the MTDT with the Amplicor PCR on the same specimens. In the Ichiyama study, the sensitivity and specificity of the MTDT were somewhat better than those obtained with Amplicor, but in the Vuorinen study, the results with the two test kits were similar (Tables 4 and 5).

The QβRA has been applied on a limited scale only (5, 174). The test is performed on a large volume of sputum, but the purification of the hybridized probe from the reaction mix is labor-intensive. PCR inhibitors do not interfere with the QβRA, but the procedure is very prone to amplicon contamination. In a study by Shah et al. on 261 sputum samples (174), the results were not superior to those of other amplification reactions: the sensitivity and specificity were 97.1 and 96.5%, respectively, and after revision were 97.3 and 97.8%, respectively.

Application of LCR (88) and NASBA (209) to tuberculosis has as yet been insufficiently evaluated.

(iv) Specimens other than sputum. PCR does not solve the problem of the bacteriological diagnosis of tuberculosis in children who do not produce sputum. Pierre et al. (153) performed a PCR on 58 gastric aspirates, for which the classical procedures are known to have a low sensitivity. When DNA amplification was applied to two gastric aspirates from the same patient and amplified in duplicate, 25% of the specimens produced at least one positive result; when three different specimens from the same subject were examined twice, the positivity increased to 60% (in 9 of 15 children).

TABLE 6. Results of MTDT for the detection of *M. tuberculosis* in smear positive and smear negative specimens

Study (reference)	MTDT sensitivity (%) in different studies		
	Overall	Smear and culture positive	Smear negative, culture positive
Abe et al. (1)	92	100	70
Bodmer et al. (12)	71	100	14[a]
Jonas et al. (92)	82	100	54[b]
Miller et al. (126)	91	94	63
Pfyffer et al. (154)	95	100	80[b]
Portaels et al. (156)	86[c]	89	85
	97[d]	97	100

[a] 86% of these were positive only in liquid medium.
[b] ≤100 CFU/ml in culture.
[c] Belgian population.
[d] African population.

The diagnosis of tuberculosis by detection of *M. tuberculosis* in peripheral blood mononuclear cells, even by a molecular amplification technique, is still impractical (164), although there has been one promising study (171). The technique is more sensitive, although not optimal, in human immunodeficiency virus-infected patients, particularly in the presence of disseminated disease (50).

Since the lack of sensitivity is the main shortcoming of the amplification techniques and the specificity is more satisfactory, the tests can be useful for organism identification. When culture in a liquid medium is combined with automated growth detection and an amplification method, the time for the diagnosis of *M. tuberculosis* can be shortened to a mean of 14 days (52). PCR and MTDT assays on clinical specimens may also be useful when there is a need for rapid differentiation between *M. tuberculosis* and nontuberculous mycobacterial infections, such as in AIDS patients in industrialized countries (172).

(v) Critique of published studies. The published studies illustrate some shortcomings in design as well as in analysis. There should be no mixtures of respiratory and other specimens, and specimens from patients being treated should not be included. Mycobacterial DNA can be detected for a long time after the start of treatment and in the absence of positive cultures in human (75) and experimental (39) models of tuberculosis. Specimens should be divided, and each portion should be prepared independently for culture and amplification. Some patients may produce sputum with unequally distributed bacilli and/or may not excrete them continuously, and the decontamination procedures may kill variable proportions of the organisms; therefore, three specimens per patient, collected at different times or days, should be examined by each method. A definition of positivity, based on microbiological rather than clinical evidence, should be established. Culture-negative, amplification-positive specimens should be retested by an amplification reaction targeted at an alternative nucleic acid fragment to reveal false-positive results, as done by Herrera and Segovia (78). The sensitivity of the amplification method should be calculated for both the number of positive specimens and the number of positive patients.

(vi) Conclusions concerning amplification techniques for diagnostic purposes. At present, the conclusions published by the Centers for Disease Control and Prevention in 1993 (23) are still valid: a particular technique cannot be replaced by a

different one if the latter is not at least equivalent to the former and at most has the same cost. At present, amplification methods for *M. tuberculosis* cannot replace the conventional diagnostic techniques, especially since strains should still be cultured for susceptibility testing. The decision of the U.S. Food and Drug Administration is equally justified: use of the rapid MTDT should be restricted to smear-positive samples from untreated patients with tuberculosis and used only in conjunction with traditional sputum examination. It should not be used for smear-negative sputum samples or for other specimens such as pleural or cerebrospinal fluid (53).

(vii) Amplification techniques for *M. tuberculosis* drug susceptibility tests. Because the molecular basis of rifampin resistance is known (97, 189, 190, 212), up to 97% of the rifampin-resistant strains can now also be identified by PCR (35, 48, 83, 211). There is one important limitation to this test: it does not measure the proportion of rifampin-resistant mutants among the isolated strain. Only when the proportion is higher than 1% is the corresponding disease resistant to rifampin therapy. Only further studies will determine how frequently isolates with a low proportion of rifampin-resistant mutants are detected by this technique. Since rifampin resistance develops mostly in isolates that are already isoniazid resistant, the recognition of rifampin resistance lends a high suspicion of multidrug resistance.

Cultures remain necessary to identify rifampin-resistant strains not detected by the PCR, to test for susceptibility to other drugs, and to allow other investigations such as restriction fragment length polymorphism for epidemiologic purposes.

Fungi

Fungal respiratory infections may be due to dimorphic fungi such as *Histoplasma* spp., *Blastomyces* spp., or *Coccidioides immitis*, and they occur sporadically in defined geographic areas. We are not aware of any effort to diagnose these infections by molecular diagnostic techniques.

A second group of fungal respiratory infections are caused by ubiquitous saprophytic fungi, occur 10 times more frequently in immunocompromised individuals (204) than in non-immunocompromised persons, and are common among patients in intensive care units. *Candida albicans* and *Aspergillus* spp. are the most frequent etiologic agents (204), and mixed infections with bacteria and cytomegalovirus occur in a significant proportion of cases. To shorten the time required for diagnosis, amplification reactions have been developed. Amplification targets have been genes coding for specific proteins (30, 95, 161, 186), 18S rDNA (15, 81, 82, 121, 124, 144), the 26S intergenic spacer region (180), or mitochondrial DNA (127). The last two represent repeated sequences, and thus their use increases the test sensitivity. In their work, Bretagne et al. (15) constructed an internal control. In some studies, primers were directed at a limited number (161) or a wide range of species; in the latter case, this was followed by treatment with restriction enzymes to obtain group identifications (82, 121, 173).

Molecular diagnostic techniques have been applied on BAL specimens and protected brush specimens to shorten the time for diagnosis, and on blood (26, 81, 128, 155) and/or urine (161) specimens in an effort to obtain a diagnosis through less invasive procedures. Only a few preliminary tests on detecting *Aspergillus* spp. in urine specimens have been performed (161). *C. albicans* was detected in seeded blood specimens (18, 81, 128), in blood samples from experimentally infected animals (95, 200), and in human blood in one study (95). The sensitivity of the PCR for *C. albicans* was disappointing: 79% (95), 73% (26), and 46% (158). Two possible reasons for this lack of sensitivity have been mentioned: the difficulty in releasing DNA from *C. albicans* cells, a critical need when they are present in small numbers (162); and the small volume of the specimen used in the amplification reaction (158). PCR has been used more frequently for classification and identification of *Candida* spp. than for their detection (93).

Spreadbury et al. (180) obtained a low sensitivity (80%) and specificity (72%) for the detection of *Aspergillus fumigatus* in clinical specimens, while Bretagne et al. (15), investigating a series of 55 specimens, obtained 25% false-positive results, i.e., detection of amplicons specific for *Aspergillus* spp. in immunocompromised patients who did not develop aspergillosis during follow-up. The authors point out numerous possibilities for contamination by environmental fungi during the preparation and storage of the reagents and the collection, transport, and manipulation of the specimens. Furthermore, the unsolved problem in the investigation of respiratory specimens for yeasts and molds is to distinguish between colonization and infection (15, 124, 134, 186). This differentiation might be possible in the future if genes related to virulence or invasiveness could be identified. At present, molecular diagnostic techniques do not improve the diagnosis of fungal infection by classical procedures.

Pneumocystis carinii

Several studies have confirmed the greater sensitivity of PCR over immunofluorescence for the detection of *Pneumocystis carinii* (22, 42, 46, 101, 111, 142, 185). Although the specificity of the assays is usually high, in one study *P. carinii* was detected in the absence of clinical symptoms (46). This could mean that colonization by *P. carinii* may occur, if contamination of samples in this study can be excluded. The conclusion of Tamburini et al. (185) that *P. carinii* should be sought in BAL specimens by the classical immunofluorescence microscopic technique and that amplification methods should be used only in exceptional cases, when the classical method remains negative, seems reasonable. In the presence of a high clinical suspicion of disease, PCR may have some utility, since claims have been made concerning the detection of *P. carinii* in sputum and two-thirds of blood specimens from patients with a generalized infection (113).

CONCLUSION

The statement that molecular diagnostic techniques, particularly PCR, are able to detect and amplify specifically a single molecule in solution in an olympic-sized swimming pool is nice but also illustrates one of the main difficulties of the procedure: how to introduce the contents of the swimming pool, or the one molecule it contains, into a 2-ml amplification vial.

The main problems facing molecular diagnostic techniques are the false-positive and false-negative results. The former may be avoided by the use of the correct controls in optimal working circumstances, i.e., good laboratory practice (177). Furthermore, any new or unusual findings should be confirmed by an independent amplification reaction. Laboratories engaging in molecular diagnostic techniques should first attain a proficiency level that excludes contamination.

Only when this technical level is reached is it possible to tackle the next problem—the test sensitivity. Much work remains to be done on this aspect. The sensitivity of use of oropharyngeal swabs and nasopharyngeal aspirates for the recovery of pathogens should be compared.

The unknown nature of most inhibitors in clinical specimens

certainly does not facilitate the development of techniques to eliminate them. Efforts to increase the sensitivity of a test by increasing the sample volume in the reaction mixture may increase the interference by inhibitors in some tests but apparently not in others. The extent to which procedures intended to concentrate the amplification target also concentrate inhibitors is unknown, as is the amount of target nucleic acid that is lost during procedures intended to eliminate inhibitors. The latter quantity could be determined by the addition of specific positive internal controls. New applications of amplification reactions should not be introduced without inclusion of specific positive internal controls. An optimal sample preparation method should be simple and rapid, and its ability to concentrate the target and eliminate inhibitors should not be nullified by its being too elaborate and time-consuming.

Compared with classical methods, nucleic acid amplification techniques are definitely more sensitive for the detection of some respiratory disease agents, particularly rhinoviruses, coronaviruses, *B. pertussis*, *M. pneumoniae*, and *C. pneumoniae*. These techniques are indispensable, not only for epidemiological studies but, for the last two organisms, also for clinical diagnostic purposes. However, in view of the results obtained in studies of other organisms, in which the sensitivity of the molecular diagnostic techniques is suboptimal, it can be surmised that the results for these agents are impressive only because the classical methods are particularly insensitive.

The great enthusiasm aroused by molecular diagnostic techniques in the field of tuberculosis detection should be tempered by the knowledge that the expectations concerning their high sensitivity and specificity have not yet been fulfilled. These problems must be addressed before amplification techniques can replace the classical diagnostic techniques. The lack of sensitivity of PCR for *M. tuberculosis* could result from the use of very small sample volumes in the reactions and an irregular dispersion of the organisms in paucibacillary samples. These shortcomings suggest the need for improved sample preparation techniques or the performance of more than one test on each sample.

The introduction of amplification techniques into the clinical diagnostic laboratory is also affected by the staff and space available and, if the decision is made to introduce them, whether they will be added to or replace existing procedures.

In conclusion, laboratories can apply molecular diagnostic techniques only if they comply with stringent external quality control requirements. As far as respiratory disease agents are concerned, amplification procedures should be limited to those listed above for which traditional culture methods are very insensitive and, depending on the geographical location, *Coxiella burnetii* and *Chlamydia psittaci*. For *M. tuberculosis*, they may be useful in some cases when an urgent identification is required if used in conjunction with culture in liquid medium and automated growth monitoring and for the rapid detection of most rifampin-resistant, and hence multiresistant, *M. tuberculosis* isolates.

We think that molecular diagnostic techniques are currently at a stage analogous to that of the clinical bacteriological techniques in the 1960s, before they were improved by many studies and gradually became standardized over the next two decades.

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Diagnostic Molecular Microbiology

PRINCIPLES AND APPLICATIONS

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Library of Congress Cataloging-in-Publication Data

Diagnostic molecular microbiology : principles and applications / edited by
David H. Persing . . . [et al.].
p. cm.
Includes index.
ISBN 1-55581-056-X
1. DNA probes—Diagnostic use. 2. Diagnostic microbiology—Technique. 3. Nucleic acid hybridization. 4. Communicable diseases—Diagnosis—Laboratory manuals. I. Persing, David H.
QR69.D4D5 1993
616.9'047583—dc20 92-38523
CIP

This book was coedited and chapter 1 was cowritten by Fred C. Tenover in his private capacity. No official support or endorsement by CDC is intended or should be inferred.

Cover illustration by Tomo Narashima

Sample Preparation Methods

6

LARRY GREENFIELD AND THOMAS J. WHITE

Although there has been progress in simplifying the release and purification of bacterial or viral nucleic acids from clinical specimens, many research procedures are still unsuitable for the clinical laboratory and a universal automated method for use with any specimen has not yet been devised. In this chapter, we review some of the basic principles that have been learned to date which may guide and encourage the reader to develop further improvements that eliminate the requirements for hazardous solutions, centrifugations, and multiple steps. A variety of approaches which may be appropriate for certain specimens and pathogens but not for others are then described. Finally, each specimen type (e.g., whole blood, urine, sputum) is discussed with regard to specific protocols and pathogens.

Basic Principles

The ideal sample preparation method represents a trade-off between the requirements for the optimal method, the clinical specimen, and the target (Table 1). Although many of these considerations are interrelated, selection of a few crucial items helps define many others. Once the target organism is selected, the clinical pathogenesis of the infection generally dictates the appropriate specimen and number of microorganisms likely to be present. Determination of the desired assay sensitivity and the number of tests to be performed on the processed sample then dictates the required volume of specimen to be processed.

Sample Size Versus Target Copy Number

Microbiological culture as a "gold standard" has directed our selection of the appropriate specimen for many infectious diseases, e.g., blood or plasma for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) and endocervical swabs for chlamydiae. For other pathogens, e.g., *Borrelia burgdorferi*, the optimal specimen for diagnosing each stage of infection has not yet been identified. For molecular diagnostic tests that are based on amplification, a single copy or molecule of the genetic target from the pathogen, if present in the reaction, can be detected in a fully optimized procedure (51, 70). To maximize the chance of diagnosing an infection, the largest convenient sample volume should be screened. However, since typical molecular diagnostic test reaction volumes are 100 μl or less, one is faced with a choice between complex target concentration steps (e.g., ethanol precipitation, nucleic acid target capture, and centrifugation) and lowered assay sensitivity. Hence, if no amplifiable target is detected

Table 4. Fundamental goals of sample preparation protocols

Release of nucleic acid from bacteria, viruses, or fungi
Stabilization of nucleic acid against degradation
Removal of amplification inhibitors
Concentration of target into a small volume
Placement of target into an aqueous environment compatible with amplification

once an active infection has cleared. A study of the course of bacteremia by culture of specimens following oral trauma showed that viable bacteria were rapidly cleared from the blood (40). In one study of chlamydia infections following treatment, results of PCR and culture showed perfect concordance (23). However, these may be best-case scenarios; other studies have documented late persistence of nucleic acid (64). In some cases it is beneficial to detect dead microorganisms, e.g., when an inaccessible reservoir of live organisms sheds bacteria that are rapidly cleared. Many more longitudinal studies of treatment to cure for various diseases will be necessary to determine the clinical significance of DNAemia.

Overview of Approaches

Sample preparation methods can be divided into a number of generic steps (Table 4). The requirement for each step will depend on the organism and specimen. The release of nucleic acid may be easy for viruses and some bacteria (e.g., *Mycoplasma* species) but difficult for other bacteria (e.g., *Mycobacterium tuberculosis*) and fungi. RNA is more difficult to stabilize than DNA. More steps may be required to remove inhibitors from some specimens (e.g., sputum and blood) than from others (e.g., urine and CSF). Some specimens (e.g., sputum for *M. tuberculosis* and *Legionella pneumophila* and blood for sepsis) may require a greater degree of concentration than others (e.g., urethral swabs and urine for *Chlamydia* or *Gonococcus* species) to achieve the required sensitivity.

There are a variety of methods for the release of nucleic acid from microorganisms, including boiling in distilled water or PCR buffer (76), detergents with or without heat (76), sodium hydroxide with heat (13), freeze-thaw (15), SDS-proteinase K (51), perchloric acid (76), enzymes (30), sonication (15), and heat (55). Enzymatic digestion may be less desirable in that there may be components in the sample which prevent the action of the enzyme. For example, lysozyme has been used on liquified sputum (30), but sputum has a high content of mucopolysaccarides. Lysozyme is unstable following reduction (77) and forms complexes with dextrans and proteins (62). Many of the current and anticipated protocols require some method of separation for concentration of nucleic acid or removal of amplification inhibitors. Potential methods for separation include centrifugation, separation by magnetic particles, and separation by filtration.

Crude Lysis

The simplest sample preparation method would entail only a crude lysis. However, such a method would require a high concentration of target in the specimen and/or small amounts of amplification inhibitors. If no other separation step is included, the volume of the specimen to be processed is limited by the volume of the amplification reaction. Simple lysis methods typically use detergents such as SDS or Triton X-100, chaotropes such as guanidinium isothiocya-

nate or sodium iodide, proteases such as proteinase K (which must be inactivated before the sample is added to the diagnostic reaction mixture) (42), substances such as saponin which lyse erythrocytes and leukocytes (e.g., the Wampole Isostat Microbial System), or heat (33). Such methods are generally suitable when the clinically significant number of infectious organisms per sample volume is large (e.g., *Chlamydia trachomatis* in endocervical swabs), so that the lysed specimen does not require significant further dilution (57). If the level of target is low, it is frequently necessary to remove amplification inhibitors by additional extraction (phenol-chloroform) steps or concentration of the target by alcohol precipitation. In addition, detergents are known to inhibit many enzymes, and high temperatures may result in degradation of nucleic acids (29).

Target Capture

Target capture or cycling offers the possible advantages of automation, universality for all specimens, and concentration of target into a small volume. This approach has been investigated by Gillespie et al. (36), Hunsaker et al. (44), and Lanciotti et al. (54). However, to date there are no published studies that demonstrate efficient capture and detection of fewer than 100 target molecules, and automated instruments and reagents for this approach are not yet commercially available. Derivatized magnetic particles can be coupled to oligonucleotide capture probes and combined with manual washing steps to remove extraneous materials (2, 19). These approaches have their own problems, though, since manual washing causes aerosols that may result in sample-to-sample contamination.

Other matrices have been tested for general adsorption of nucleic acids. Glass matrices, Sephadex, and diatomaceous earth bind nucleic acids in chaotropic solutions (11, 16, 59, 79). Following binding of the nucleic acids to the solid-phase matrix, the impurities and amplification inhibitors are removed by centrifugation and washing and the nucleic acids are eluted in an amplification-compatible buffer. Such approaches are promising since they are relatively simple, can be automated, and do not require hazardous reagents.

Finally, filtration may become a useful approach for certain kinds of specimens if it can be automated and made rapid (7a). Cost will be a problem unless disposable devices can be manufactured cheaply, and the requirement for a vacuum or centrifuge could be a burden for many laboratories.

Recommended Protocols for Various Specimens

Whole Blood

Even after it is decided that the desired specimen for a given target is blood, there still remain a number of choices: plasma, serum, whole blood, leukocyte fractions, etc. Furthermore, there is a choice of anticoagulants if the specimen is plasma: EDTA, heparin, or citrate. The anticoagulant used for plasma collection and the method of storage may affect the ability of the assay to detect the presence of target sequences (17, 80). Heparin was found to inhibit the activity of both murine leukemia virus reverse transcriptase and *Taq* DNA polymerase (46). In addition, the inhibitory effect of heparin does not appear to be removed by extraction of RNA by a modification of the acid-phenol-guanidinium method. For EDTA-containing tubes, it is recommended that the final concentration of EDTA be 1 to 2 mg/ml of blood (final concentration, 6.8 mM). For heparin-containing

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SANDWICH HYBRIDIZATION METHOD FOR NUCLEIC ACID DETECTION

This invention relates generally to the field of nucleic acid technology and specifically, relates to methods for detecting specific genes or base sequences on single stranded nucleic acid.

The recent development of genetic engineering and in particular, the manipulation of nucleic acids and genes in order to force production of desired metabolites, enzymes and the realization certain disesse ststes sre governed by identifisble base sequences, has created a demand for methods for detecting the presence of desired (or undesired) genes or base sequences in nucleic acids.

It is an object of the present invention to provide sensitive methods for the detection of such genes or base sequences.

Recently, Ranki et al. described in Gene, 21:77-85 (1983) a DNA probe sandwich assay useful for detecting a specified portion of a DNA strsnd. To do so, one probe is immobilized on nitrocellulose and is used to capture the sample DNA (deoxyribose nucleic acid) by hybridizing with the specific gene under investigation. Ranki also provides a second probe which is labeled and also nonspecific to the gene of interest.

Ranki's method, however, suffers from several disadvantages including variations in specific and nonspecific binding characteristic of extended solid support systems such as those employing nitrocellulose. The repeatability of such solid support preparations has

been shown to be a key difficulty in the production of coated macroscopic surfaces such as coated test tubes in the field of immunoassays. These variations ultimately limit the sensitivity of the assay to the range of the variations. Accordingly, it may be expected that similar problems will be associated with Ranki's method.

It is an object of the present invention to avoid these limitations by providing a method capable of more repeatable commercial production.

Summary of the Invention

In accordance with the objectives of the present invention, methods are provided for detecting nucleic acids having a desired gene or base sequence comprising the steps of providing the nucleic acid to be tested in a single stranded form and thereafter contacting it with a labeled nucleic acid probe specific for a given section of the nucleic acid strand. A biotinylated nucleic acid probe specific for a different portion of the nucleic acid strand, is bonded to an avidin coated microparticle. The strand having the labeled probe hybridized thereto is then mixed with the thusly prepared microparticles. Binding of the DNA strand to the microparticle occurs through the biotinylated probe that coats the microparticle. The avidin-biotin coupling is sufficiently strong to permit the separation of microparticle bound DNA from unbound material. The bound material is subsequently assayed for label. The presence of the label is indicative of the presence of a strand having both portions for which the biotinylated and labeled probes are specific. Either one of these

sections may be the gene or base sequence of interest. Alternately, the decrease of label in solution may be monitored and related to the presence of the gene or base sequence of interest.

Brief Summary of the Drawing

In accordance with the principles of the present invention, further understanding may be had by reference to the figure wherein:

FIG. 1 artistically shows the labeling and immobilization of a single stranded nucleic acid.

Detailed Description of the Drawing and Best Mode

With reference to FIG. 1, single stranded nucleic acid 10 is provided and may be in the form of ribose nucleic acid or deoxyribose nucleic acid which has at least been reduced to a single stranded form by any of a number of well known techniques such as heating or adding a strong base. The nucleic acid strand 10 is reacted with both a nucleic acid probe 11 having a label 12, and a biotinylated nucleic acid probe 13. These reactions will be carried out in solution and preferably with an excess of labeled nucleic acid probe 11.

Also provided are microparticles 14 coated with avidin. The microparticles may be made from a variety of materials including glass, nylon, polymethacrylate, other polymeric material, or biological cells. Such microparticles may be readily obtained from a variety of sources including, for instance, Polysciences Inc.,

Pennsylvania. An avidin coating may be readily prepared by physical adsorption or direct chemical means such as coupling via the N-hydroxysuccinimide active ester (Manning et al., Biochemistry 16:1364-1370, 1977) in the case of glass, or a carbodiimide coupling in the case of nylon (Jasiewicz et al., Exp. Cell Res. 100:213-217, 1976).

If it is desired to avoid the use of avidin-biotin coupling mechanisms with microparticles, one may instead employ nitrocellulose microparticles, as opposed to Ranki's nitrocellulose sheets, to obtain direct chemical bonding of single stranded nucleic acid to the solid phase. Although the mechanism of attachment is as yet unknown, it has been learned that after attachment of the nucleic acid, remaining attachment sites must be blocked by reaction with additional DNA such as salmon testes DNA prior to utilization of the thusly prepared solid phase surface.

The mixture of nucleic acid having biotinylated probe hybridized therewith with the avidin coated microparticles or beads will, due to the strong avidin-biotin attraction, result in the immobilization of the nucleic acid hybrid pair. The nucleic acid may then be separated from the unbound materials by centrifugation, filtration, or washing steps such as those that may be employed with heterogeneous immunoassay techniques.

The label will preferably be selected in order to be optically detectable with readily available instruments for yielding acceptable sensitivities. Such labels will

include, for instance, fluorophores, i.e. those that fluoresce under suitable excitation wavelengths, chemiluminescent labels which luminesce under appropriate chemical conditions, or any of the enzyme associated labeling mechanisms whereby a detectable product is produced by the action of an enzyme label upon a substrate. Such a product may, for example, be selected so as to be readily detected colorimetrically. Although less preferred, the label may instead be a radioisotope. Typically, however, isotopic labels are less preferable as they require special handling due to significant health risks associated with such labels, exhibit finite shelf-life, and require expensive equipment such as scintillation or gamma counters. Still other types of labels are contemplated and would include for instance, materials possessing detectable electromagnetic properties.

In addition to the microparticles described above, it is also contemplated that the methods provided herein may be employed with other types of solid phase surfaces including, for instance, the walls of a microtiter tray, paddles, or other macroscopic surfaces such as disks or tapes. Such disks or tapes may have the avidin coatings arranged thereon in a pattern. Such patterns are useful, for example, in the class of instruments employing synchronous detection.

Various well known techniques are available for coupling labels with nucleic acid probes. One such technique is described by Langer et al. in Proc. Natl. Acad. Sci. 78:6633-6637, 1981.

A preferred order of reaction employing avidin and biotin to couple label 12 to probe 11 would involve the following steps. First, biotinylated probe 11 is reacted with nucleic acid strand 10 followed by reaction of avidin coupled label 12 with biotinylated probe 11 to form a first mixture. Separately, avidin coated microparticles 14 are reacted with biotinylated probe 13 to form a second mixture. The first and second mixtures are reacted to permit formation of complexes as depicted in FIG. 1. These immobilized complexes are then separated and either the label associated therewith or the free label remaining in the solution measured. An example of a nonpreferred order of reaction would permit binding of a biotinylated probe 11 with an avidin coated microparticle 14.

The order of reaction between the single stranded nucleic acid, the biotinylated nucleic acid probe (not employing avidin-biotin), the labeled nucleic acid probe, and the avidin coated solid phase support may be varied in order to suit the needs of the investigator. For instance, it may be found desirable to react the biotinylated nucleic acid probe with the avidin coated solid phase surface prior to adding the nucleic acid strand. The nucleic acid strand may be either previously reacted with the labeled nucleic acid probe or subsequently reacted therewith. A nonpreferred order of reaction would permit binding of a biotinylated probe 11 with an avidin coated microparticle 14.

In another embodiment, it is contemplated that one may wish to provide microparticles coated with biotinylated nucleic acid probe segments useful for any assay. These

microparticles could then be customized for any given assay by reacting a longer nucleic acid probe, or linking probe, with the biotinylated probe so that the section of the linking probe that is homologous to the biotinylated probe forms a double stranded nucleic acid section. The remaining portion of the linking probe would preferably be homologous to the gene being sought in the nucleic acid under analysis. Preparation of the solid phase portion in this manner, particularly with respect to microparticles, would allow manufacturing to occur on a large scale and would supply essential components useful for any nucleic acid assay.

Employment of microparticles is greatly preferred since it is to be expected that there may be significant variation of immobilized probe 13 present on microparticles 14, however, if a large quantity of microparticles 14 are prepared and randomly divided among a number of assay tests, then the variation in total probe 13 from test to test will be reduced in proportion to the reciprocal of the square root of the number of particles used per test.

The skilled investigator will readily appreciate that separation of unbound nucleic acid strands in those embodiments employing solid phase surfaces such as microtiter well walls, or macroscopic surfaces and the like, will be readily accomplished by gentle washing steps.

Nucleic acid probes such as those contemplated in the present invention, may be readily obtained from a variety of sources including Enzo-Biochem, New York, NY.

It will be readily understood by those skilled in the art that numerous alterations of the above may be had without departing from either the spirit or the scope of the present invention.

CLAIMS

1. A method for detecting the presence of nucleic acid containing a gene or base sequence of interest comprising:

a) providing the nucleic acid to be tested in a single strand form;

b) contacting a biotinylated nucleic acid probe specific for a first gene or base sequence of said nucleic acid with an avidin coated solid phase support;

c) reacting said nucleic acid with an excess of labeled nucleic acid probe specific for a second gene or base sequence of said nucleic acid and permitting any hybridization of said labeled probe and said nucleic acid to occur;

d) reacting said nucleic acid with said biotinylated nucleic acid probe and permitting any hybridization of said biotinylated probe and said nucleic acid to occur;

e) separating said unbound nucleic acid from said bound nucleic acid; and

f) detecting the label associated with said bound nucleic acid or the decrease in the amount of label in solution for determining the presence of nucleic acid containing the gene or base sequence of interest.

2. The method as provided in Claim 1 wherein the order of reaction is as provided in Claim 1.

3. A method for detecting the presence of nucleic acid containing a gene or base sequence of interest comprising:

a) providing the nucleic acid to be tested in a single stranded form;

b) further providing an avidin coated solid phase support which has been reacted with a first biotinylated nucleic acid probe to form a first solid phase species;

c) contacting said first solid phase species with a nucleic acid probe linking means having a first portion which is reactive with at least some part of said first biotinylated nucleic acid probe and a second portion reactive with a first gene or base sequence of said nucleic acid and permitting any hybridization to occur to form a first mixture;

d) reacting said nucleic acid with an excess of labeled nucleic acid probe specific for a second gene or base sequence of said nucleic acid and permitting any hybridization of said labeled probe and said nucleic acid to occur to form a second mixture;

e) reacting said first mixture with said second mixture;

f) separating solid phase immobilized nucleic acid from unbound nucleic acid; and

g) detecting the label associated with said immobilized nucleic acid portion for

determining the presence of nucleic acid containing the gene or base sequence of interest.

4. The method as provided in Claim 3 wherein the order of reaction is as set forth in Claim 3.

5. The method of any one of Claims 1 to 4 wherein the solid phase is selected from the group consisting of microparticles and macroscopic surfaces.

6. The method as provided in Claim 5 wherein the solid phase comprises microparticles.

7. The method as provided in Claim 5 wherein the solid phase comprises macroscopic surfaces.

8. The method as provided in Claim 7 wherein said biotinylated label is immobilized on said macroscopic surface in a specific spatial pattern.

9. The method of any one of Claims 1 to 8 wherein the detecting step comprises detecting fluorescence.

10. The method of any one of Claims 1 to 8 wherein the detecting step comprises detecting chemiluminescence.

11. The method of any one of Claims 1 to 8 wherein the detecting step comprises detecting an isotopic label.

12. The method of any one of Claims 1 to 8 wherein the detecting step comprises detecting products of enzymatic labels.

13. The method of any one of Claims 1 to 8 wherein the detecting step comprises detecting light scatter.

14. The method of any one of Claims 1 to 4 wherein the detecting step comprises detecting by colorimetry.

15. The method of any one of Claims 1 to 14 wherein the providing step further comprises splitting double stranded nucleic acid into single stranded nucleic acid if said nucleic acid is deoxyribose nucleic acid.

16. A method for detecting the presence of nucleic acid containing a gene or base sequence of interest comprising:

a) providing the nucleic acid to be tested in a single strand form;

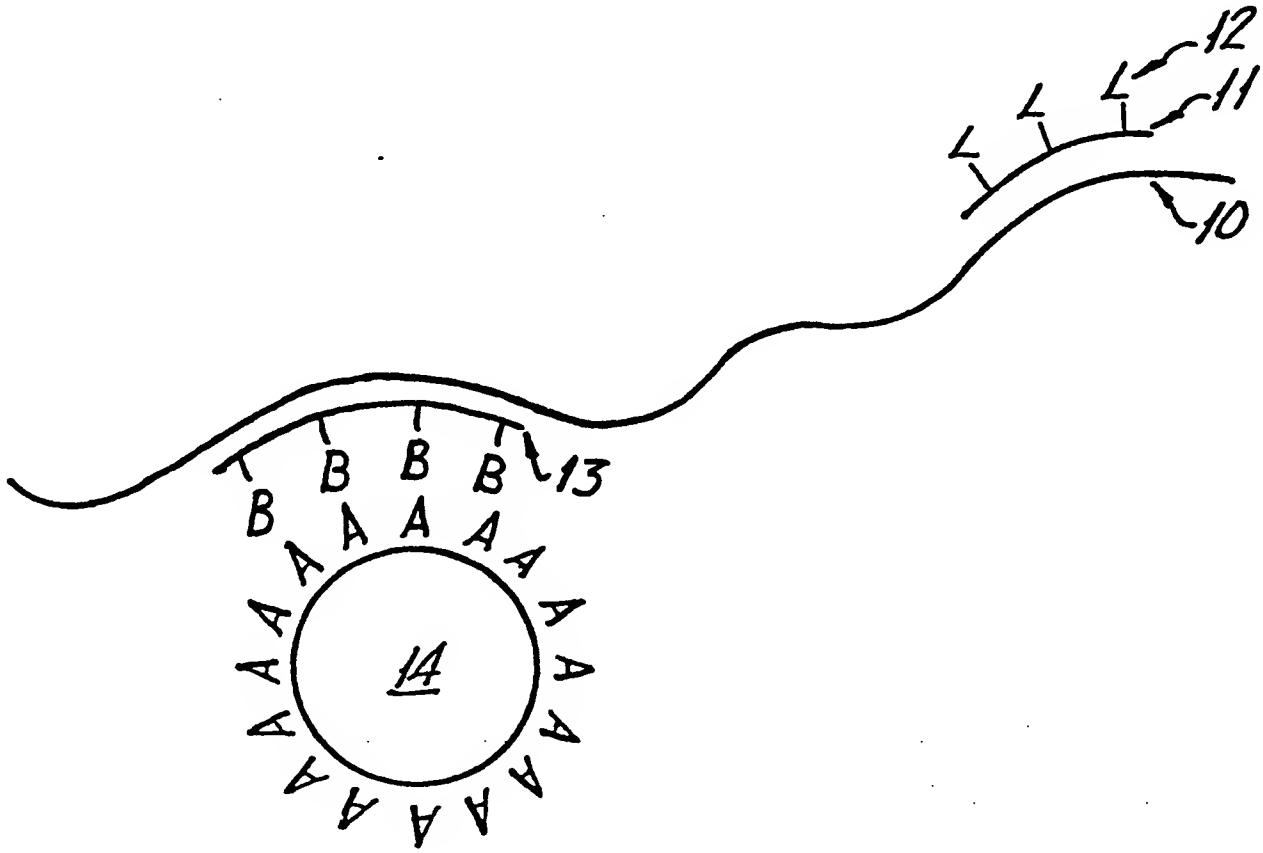
b) attaching a nucleic acid probe specific for a gene or base sequence of said nucleic acid to a nitrocellulose microparticle;

c) reacting said nucleic acid with an excess of labeled nucleic acid probe specific for a first gene or base sequence of said nucleic acid and permitting any hybridization of said labeled probe and said nucleic acid to occur;

d) reacting said nucleic acid with said nitrocellulose attached nucleic acid probe and permitting any hybridization to occur;

e) separating said unbound nucleic acid from said bound nucleic acid.

f) detecting the label associated with said bound nucleic acid or the decrease in the amount of label in solution for determining the presence of nucleic acid containing the gene or base sequence of interest.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JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1209–1215
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Vol. 34, No. 5

Sequence Capture-PCR Improves Detection of Mycobacterial DNA in Clinical Specimens

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Received 22 November 1995/Returned for modification 3 January 1996/Accepted 22 February 1996

The rapid identification of mycobacterial DNA in clinical samples by PCR can be useful in the diagnosis of tuberculous infections, but several large studies have found that the sensitivity of this approach is not better than that of culture. In order to improve the sensitivity of detection of mycobacterial DNA in clinical specimens from patients with paucibacillary forms of tuberculosis, we have developed a procedure permitting the specific capture of mycobacterial DNA in crude samples prior to amplification, thereby concentrating the target sequences and removing irrelevant DNA and other potential inhibitors of the amplification reaction (sequence capture-PCR). By using this approach to capture and amplify two different sequences specific for organisms of the *Mycobacterium tuberculosis* complex (IS*6110* and the direct repeat region), it was possible to detect as little as one genome of mycobacterial DNA in samples containing up to 750 μg of total DNA, representing a 10- to 100-fold increase in sensitivity compared with that obtained by purifying total DNA prior to amplification. Detection of the IS*6110* sequence in pleural fluid samples from patients with tuberculous pleurisy by sequence capture-PCR gave positive results in 13 of 17 cases, including 3 of 3 culture-positive samples and 10 of 14 culture-negative samples. In contrast, when total DNA was purified from these samples by adsorption to a silica matrix prior to amplification, only the three culture-positive samples were positive by PCR. The sensitivity of detection of the direct repeat sequence in these samples by sequence capture-PCR was similar to that of IS*6110* and, in addition, permitted immediate typing of the strains from some patients. We conclude that sequence capture-PCR improves the sensitivity of detection of mycobacterial DNA in paucibacillary samples. This approach should be useful in detecting rare target sequences from organisms implicated in other pathologic processes.

Tuberculosis remains a major worldwide health problem and, because of its protean manifestations, must be considered in the differential diagnosis of numerous patients (2, 3, 15). Unfortunately, the standard methods used in the diagnosis of tuberculosis have several important limitations. Microscopic identification of acid-fast mycobacteria is insensitive and, when positive, does not permit identification of the species of mycobacterium identified. Mycobacterial culture may require several weeks to obtain positive results and frequently gives negative results for paucibacillary forms of tuberculosis. These limitations create a variety of problems in the clinical management of patients suspected of having tuberculosis and may lead to delays in initiating appropriate treatment and/or the use of invasive procedures to firmly establish or exclude this diagnosis.

In an effort to overcome these problems, a number of laboratories have evaluated the usefulness of the detection of mycobacterial DNA in clinical samples by techniques based on PCR in the diagnosis of tuberculosis. Several large studies have found that this approach can be used to rapidly diagnose tuberculous infections with a sensitivity that is equivalent to or somewhat less than that of mycobacterial culture (7, 8, 12, 13, 21, 25, 28). Unfortunately, most studies have found that not all samples which are direct examination negative and culture positive are also positive by PCR and that only a minority of culture-negative samples from patients ultimately shown to have tuberculosis are positive by this approach. Thus, in clinical situations in which improvements in diagnostic techniques are most needed (paucibacillary forms of tuberculosis), current PCR techniques have not been of considerable help.

Two obstacles have limited the sensitivity of this approach in the diagnosis of tuberculosis. First, the presence of too much DNA can inhibit PCR, and many clinical specimens (blood, bronchoalveolar lavage fluids, pleural fluids, bone marrow aspirates, tissue biopsies, etc.) contain large numbers of immune and inflammatory cells, a source of large amounts of DNA. Thus, it is necessary to dilute these samples (and consequently the mycobacterial DNA present) prior to amplification. Second, to obtain optimal sensitivity, it is necessary to eliminate inhibitors of the amplification reaction present in clinical samples. Unfortunately, the multistep processes required to obtain highly purified DNA are difficult to apply in routine practice.

To overcome these problems, we have developed an approach that permits the specific capture of mycobacterial DNA in crude samples containing large numbers of human cells, thereby permitting the removal of irrelevant DNA and potential inhibitors present in the original sample prior to amplification. Using this technique, we have demonstrated that this enrichment leads to the anticipated increase in the sensitivity of detection of mycobacterial DNA in standard samples containing known amounts of mycobacterial DNA and in paucibacillary clinical samples from patients with tuberculous pleurisy.

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MATERIALS AND METHODS

Materials. The oligonucleotides used for amplification of a 123-bp fragment of the IS*6110* insertion element (IS1 and IS2) and the direct repeat (DR) region of *M. tuberculosis* (DRa and DRb) have been previously described (11, 16, 23). Oligonucleotides IS3 (13) and DRc (5'-CCCAAAACCCCGAGAGGG) were used for the detection of amplification products by Southern blotting. Capture oligonucleotides for the IS*6110* sequence were Cap-1, 5'-AAAAACGAACG GCTGATGACCAAACTC, and Cap-2, 5'-AAAAAGGAGGTGGCCATCGT GGAAG. These oligonucleotides are complementary to IS*6110* sequences 97 bases upstream of that recognized by IS1 and 39 bases downstream of that recognized by IS2 and therefore do not recognize products amplified by IS1 and IS2. The oligonucleotides were positioned to hybridize with regions devoid of inverted repeat sequences identified by using the STEMLO program. Because the repetitive sequence in the DR region is only 36 bp long, the oligonucleotides used for the capture of DR sequences were identical to the oligonucleotides, DRa and DRb, used to amplify this region, except that 5 adenosine residues were added to the 5' ends. All oligonucleotides were synthesized by Genset (Paris, France). Capture oligonucleotides were synthesized with a biotinylated 5-carbon spacer arm attached to the 5'-end and were purified by high-pressure liquid chromatography. In preliminary experiments evaluating the efficiency of capture of biotinylated oligonucleotides by avidin-coupled magnetic beads, capture oligonucleotides were labelled at their 3' ends with [α-^{32}P]dCTP (Amersham, Slough, United Kingdom) by using terminal deoxytransferase (10).

To evaluate the presence of inhibitory substances in amplification reactions, an internal standard in which the sequences recognized by IS1 and IS2 were added to opposite ends of a 403-bp fragment of plasmid pGEM-3 and which generated a 443-bp fragment when amplified by primers IS1 and IS2 was constructed. Serial dilutions were tested, and the last dilution which gave consistently positive results when amplified in the presence of 0.5 μg of highly purified human DNA (5 μl of a 10^{-8} dilution) was used to verify that specimens could support amplification.

DNA from *M. tuberculosis* H37Rv was purified and quantified by densitometry, and serial dilutions were prepared by using a solution containing 100 μg of human DNA (human placental DNA; Sigma, St. Louis, Mo.) per ml to produce standards containing 0.1 to 100 genomes per 5 μl, assuming a molecular mass of 2.5×10^9 Da for 1 mycobacterial genome (e.g., 1 genome = 3 to 4 fg). To evaluate techniques used for the extraction of mycobacterial DNA, *M. tuberculosis* H37Rv was grown in suspension culture in 7H9 medium, organisms were quantified by limiting-dilution culture, and aliquots containing <10 viable organisms were added to tissues prior to DNA extraction.

Pleural fluid samples. Samples of pleural fluid also submitted for mycobacterial culture were obtained from 17 patients with tuberculous pleurisy evaluated at Hôpital Tenon, Paris, France (age, 38.6 ± 14.5 years; 13 men and 4 women). For 11 patients, the diagnosis was established on the basis of positive culture(s) for *M. tuberculosis* of samples of sputum, pleural fluid, and/or pleural biopsies. For six patients, all mycobacterial cultures were negative and the diagnosis was based on the demonstration of caseating granulomas in pleural biopsies. Cultures of pleural biopsies, performed on seven patients, were positive in four cases. None of the patients had a positive serologic test for human immunodeficiency virus, and none had any other disease known to produce immunosuppression. The volume of pleural fluid obtained from these patients was 5 to 1,000 ml (average, 185 ± 319 ml). Acid-fast staining and mycobacterial culture were performed as previously described (22), except that sputum samples were decontaminated by treatment with 4% sodium hydroxide.

To serve as controls, pleural fluid samples from 25 patients (age, 56.6 ± 15.4 years; 21 men and 4 women) without tuberculosis were also evaluated. The causes of pleural effusion in these patients were as follows: metastatic carcinoma (n = 13), mesothelioma (n = 2), parapneumonic pleural effusion (n = 8), and lymphoma (n = 2). The volume of pleural fluid obtained from these patients ranged from 8 to 1,000 ml (average, 132 ± 253 ml). In seven cases, two different aliquots of pleural fluid were used as control samples.

Solubilization of samples. Pleural fluid samples were centrifuged (2,240 × *g*; 30 min). Cell pellets or fragments of tissue biopsies were suspended in 500 μl of 100 mM Tris-HCl containing 150 mM NaCl and 50 mM EDTA (pH 7.4), and transferred to 2-ml screw-cap tubes (Eppendorf, Freemont, Calif.) containing 0.5 ml of 0.1-mm-diameter glass microspheres (Biospec Products, Bartlesville, Okla.) and 50 μl of 20 mg of proteinase K (Interchim, Montluçon, France) per ml. Samples were agitated (Mini-BeadBeater; Biospec) for 50 s, allowed to digest overnight at 50°C (Thermomixer; Eppendorf), and agitated again for 50 s, and the supernatant (crude extract) was recovered by centrifugation. Preliminary experiments performed with samples containing small numbers of intact mycobacteria demonstrated that this procedure was highly efficient in releasing mycobacterial DNA.

The DNA in crude extracts was measured by spectrofluorometric assay, as previously described (5). An aliquot containing 5 μg of DNA was removed, and DNA was purified by adsorption to a silica matrix (Geneclean II; BIO 101, Inc., La Jolla, Calif.) as previously described (4, 12). Purified DNA was eluted from the silica matrix into 30 μl of distilled water, and 10-μl aliquots were used for amplification.

Sequence capture. Crude extracts from tissues and cells (final volume, 0.55 ml, containing up to 750 μg of total DNA) were transferred to 1.5-ml Eppendorf tubes, heated at 100°C for 10 min, and cooled to 0°C on ice, and 0.2 ml of 3.75 M NaCl–2.5 pmol each of biotinylated capture oligonucleotides Cap-1 and Cap-2 was added (final volume, 0.75 ml in 1 M NaCl). Tubes were incubated with agitation (Thermomixer) at 60°C for 3 h to allow hybridization. Ten microliters of M-280 Streptavidin Dynabeads (Dynal, Oslo, Norway), washed according to the manufacturer's instructions, was added, and the incubation was continued for 2 h at 20°C. Magnetic beads were captured (Dynal magnetic-particle concentrator), washed twice with 10 mM Tris-HCl–0.1 mM EDTA (pH 8), and resuspended in water. Two aliquots, each containing 5 μl of beads in 10 μl of water, were used for amplification. Capture of the DR region was performed by analogous techniques, except that the Cap-DRa and Cap-DRb oligonucleotides were used and hybridization performed at 42°C. The procedure is summarized in Fig. 1.

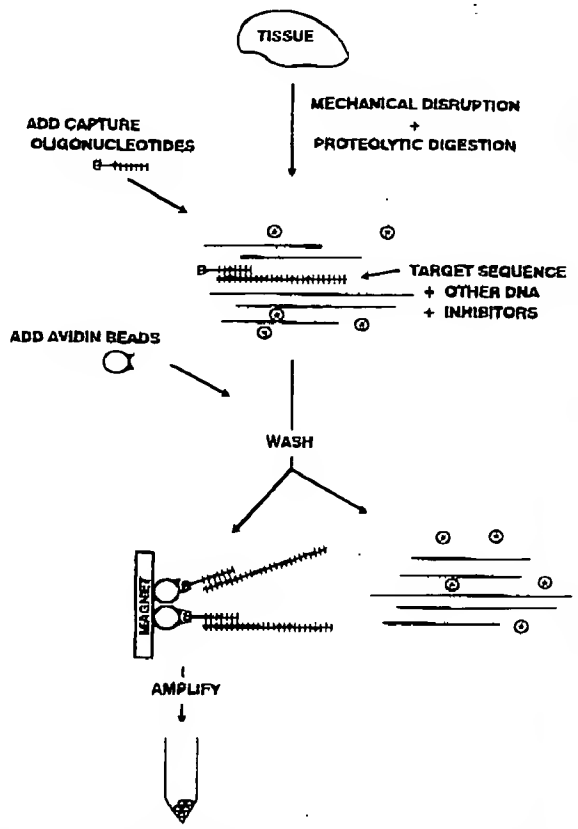

FIG. 1. Schematic representation of sequence capture-PCR. DNA is liberated from tissues or cells, producing a crude extract containing the specific target DNA sequence (hatched bar), human DNA (lines), and potential inhibitors of the amplification reaction (stars). The target sequence is specifically captured by the sequential addition of biotinylated capture oligonucleotides and avidin-coupled magnetic beads. The beads are added directly to the amplification reaction mixture.

Amplification and detection of mycobacterial DNA. Samples for amplification (see above) were suspended in a final volume of 45 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 100 μg of gelatin per ml; 0.2 mM (each) dATP, dGTP, dCTP, and dUTP; 12.5 pmol of each oligonucleotide primer; and 1 U of uracil-*N*-glycolase (Gibco BRL, Gaithersburg, Md.). Samples were incubated at 37°C for 10 min, heated to 95°C for 10 min, and cooled to 80°C in a thermal cycler (Perkin-Elmer, Norwalk, Conn.). Five microliters of a solution containing 1 U of *Taq* DNA polymerase (Appligene, Illkirch, France) was added by using a positive-displacement pipette prior to amplification. For amplification of the IS*6110* insertion element (oligonucleotides IS1 and IS2), the cycling parameters were 95°C for 40 s, 65°C for 40 s, and 72°C for 15 s for 50 cycles. For amplification of the DR region (oligonucleotides DRa and DRb), 2.0 mM $MgCl_2$ was used; the cycling parameters were 95°C for 40 s, 55°C for 40 s, and 72°C for 15 s for 50 cycles. Amplification products were electrophoresed onto agarose gels and transferred to nylon membranes, membranes were hybridized with ^{32}P-labelled oligonucleotides, and positive signals were detected by autoradiography as previously described (23).

Mycobacterial typing. To type mycobacterial DNA amplified in clinical specimens, the spacer oligotyping method described by Kamerbeek et al. (16) was used. Briefly, a 5-μl aliquot of amplification products from positive reactions were reamplified for 25 cycles by using the DR primer set in which the DRa oligonucleotide was biotinylated at the 5' extremity. Aliquots of the amplified

products were hybridized (60°C, 60 min) in a reverse line blotting assay (17) by using a membrane to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *Mycobacterium bovis* BCG had been covalently linked. Membranes were washed at 60°C to remove unbound amplification products and incubated with horseradish peroxidase-labelled streptavidin (Boehringer, Mannheim, Germany), and positive hybridization was revealed by reaction with ECL detection reagents and by exposure of ECL hyperfilm (Amersham, Hertogenbosch, The Netherlands).

Interpretation of results. In experiments evaluating clinical samples, each sample of pleural fluid from a patient with tuberculosis was processed in parallel with four control samples during all steps of the procedure (solubilization of DNA, purification of DNA by sequence capture and adsorption to silica matrix, and amplification). Two types of control specimens, spleen fragments from Wistar rats and pleural fluid samples from patients without tuberculosis, were used. Two identical aliquots of DNA purified by sequence capture or silica matrix adsorption from the same sample were amplified in all cases. Samples were considered positive if one or both of the reactions gave a positive signal on autoradiography. Statistical comparisons were made by using the χ^2 test.

RESULTS

Optimization of PCR. To minimize false-positive results due to carryover of amplified products from prior reactions, all PCRs were performed with dUTP instead of dTTP, and new reaction mixtures were pretreated with uracil-*N*-glycosylase prior to amplification (19). After optimization of reaction conditions, positive results were obtained for amplification of the IS*6110* fragment in 55 of 60 samples containing one genome of DNA from *M. tuberculosis* in 500 ng of human DNA (final volume, 50 μl), 28 of 60 samples containing as little as 0.1 genome, and 0 of 60 samples without mycobacterial DNA. This sensitivity is similar to that we obtained by amplifying this sequence with dTTP (23) and approaches the maximal theoretical sensitivity of the test. (Assuming that *M. tuberculosis* H37Rv contains 15 copies of the IS*6110* sequence and that DNA was fragmented during purification such that each sequence was on a separate fragment, 78 of 100 samples containing 0.1 genomes would contain an amplifiable target.) As previously reported (18), optimal sensitivity was strictly dependent on the total amount of DNA present. When one genome of mycobacterial DNA was added to <1 μg of human DNA, 10 of 10 amplifications were positive, but 3 of 10 and 0 of 5 reactions were positive when the same amount of mycobacterial DNA was amplified in the presence of 2 and 5 μg of human DNA, respectively.

Development of techniques for sequence capture-PCR. Because the presence of excess human DNA impairs the sensitivity of detection of mycobacterial DNA, we developed an approach to selectively purify mycobacterial DNA prior to amplification. Commonly, biotinylated oligonucleotides are attached to avidin-coated beads and subsequently incubated with denatured DNA containing sequences to be captured (direct capture). Positive results can be obtained by this approach for samples containing large amounts of mycobacterial DNA (≥100 genomes). We found, however, that direct capture rarely gave positive results for samples containing 10 or fewer mycobacterial genomes (data not shown), and this technique was abandoned in favor of the two-step capture procedure depicted in Fig. 1.

To ensure that all captured sequences are present in the amplification reaction mixture, it is desirable to directly add magnetic beads containing the captured sequences to the PCR reaction mixture. The addition of up to 5 μl of magnetic beads had no deleterious effect on the amplification of mycobacterial DNA, although larger amounts of beads had progressively prominent inhibitory effects. Thus, capture was performed with 10 μl of beads; beads were subsequently divided into two equal aliquots (5 μl each) prior to amplification. This amount of magnetic beads could completely bind up to 5 pmol of each

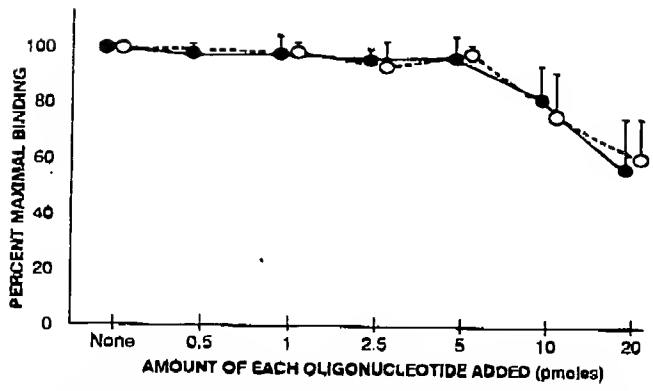

FIG. 2. Binding of capture oligonucleotides by avidin-coupled magnetic beads. Biotinylated capture oligonucleotides were labelled with ^{32}P at their 3' ends by using terminal transferase, and tracer amounts of radiolabelled oligonucleotide were incubated with 10 μl of avidin-coupled magnetic beads for 2 h at 20°C in the absence (None) or presence of the indicated amounts of each unlabelled capture oligonucleotide, Cap-1 and Cap-2 (solid symbols; $n = 4$) or Cap-DRa and Cap-DRb (open symbols; $n = 3$). Data are the means ± standard deviations of the maximum percentage of oligonucleotide bound, which represented >85% of total radioactivity.

capture oligonucleotide, but the binding of larger amounts of oligonucleotides was incomplete (Fig. 2). The efficiencies of capture of small amounts of mycobacterial DNA (≤10 genomes) by using 1 and 2.5 pmol each of the two biotinylated capture oligonucleotides were compared and found to be equivalent (data not shown). These results indicate that the use of 2.5 pmol of each oligonucleotide was sufficient to ensure that the concentration of capture oligonucleotides was not a limiting factor in the efficient capture of mycobacterial DNA.

Numerous other factors affecting the efficiency of sequence capture (e.g., solubilization of DNA, composition of the hybridization solution, and times and temperatures during hybridization and binding of oligonucleotides to beads) were also evaluated. To test the overall sensitivity of the conditions defined in these studies, fragments of animal tissues or human immune and inflammatory cells obtained by centrifugation of pleural fluid samples were digested by the established protocol and small amounts of mycobacterial DNA were added to some samples prior to performing capture and subsequent amplification of the IS*6110* sequence. In these studies, 4 of 4 samples containing 100 mycobacterial genomes, 27 of 29 samples containing 10 mycobacterial genomes, and 8 of 13 samples containing 1 mycobacterial genome gave positive results, whereas none of the samples containing no added mycobacterial DNA was positive (Table 1). The positive samples used in these studies contained up to 750 μg of DNA. Thus, it was possible to detect mycobacterial DNA in samples containing as little as 0.001 mycobacterial genome per μg of total DNA, representing a 10- to 100-fold increase in sensitivity over that obtained by amplifying samples without prior enrichment of mycobacterial DNA.

Detection of mycobacterial DNA in pleural fluid samples from patients with tuberculous pleurisy. To determine whether the improved sensitivity of the sequence capture technique would improve the detection of mycobacterial DNA in clinical samples, it was important to use specimens containing only small numbers of mycobacteria. Pleural fluid samples from patients with tuberculous pleurisy were chosen for this purpose. Compatible with the results for prior series (6, 9), mycobacteria were not observed in pleural fluid samples from patients with tuberculous pleurisy by acid-fast staining and only 3 of 17 of these samples were positive by culture (Table 2). For

TABLE 1. Comparison of the sensitivities of sequence capture-PCR in detecting two different mycobacterial sequences, IS*6110* and the DR region[a]

Type of sample	No. of samples positive/no. tested	
	IS*6110*	DR region
Animal tissues or human cells with purified mycobacterial DNA added[b]		
100 genomes	4/4	ND[c]
5–10 genomes	27/29	25/26
1–2 genomes	8/13	2/6
Pleural fluids from patients with tuberculosis[d]	11/15	10/15
Control tissues	0/34	0/25

[a] $P > 0.3$ for all comparisons between IS*6110* and the DR region by Fisher's exact test.
[b] The total DNA was ≤750 μg.
[c] ND, not done.
[d] Only samples for which sequence capture-PCR using both systems was performed.

the detection of mycobacterial DNA by PCR, fluid samples obtained by thoracentesis were centrifuged and DNA was extracted from the cell pellet by mechanical disruption and proteolytic digestion. DNA was purified from an aliquot of the sample by adsorption to a silica matrix, and the remainder of the DNA, up to a limit of 750 μg (total), was used for sequence capture (375 ± 278 μg per sample; $n = 17$).

When total DNA purified by adsorption to a silica matrix was used for amplification of the IS*6110* sequence by the IS1 and IS2 primer pair, only 3 of the 16 samples evaluated were positive; the positive samples corresponded to those that were also positive by culture. To ensure that negative samples could support amplification, an internal standard that generates a 443-bp product when amplified by the IS1 and IS2 oligonucleotides was added to an identical aliquot of each sample prior to amplification. The presence of an amplification product of the expected size was observed in 16 of 16 samples, indicating that the presence of inhibitory substances could not explain the negative results obtained with these samples.

In contrast, when DNA was enriched for mycobacterial DNA by the sequence capture technique prior to amplification of the IS*6110* sequence, positive results were obtained for 13 of 17 samples from patients with tuberculous pleurisy, including the 3 samples that were positive by culture and 10 of the 14 samples that were culture negative ($P < 0.01$; comparing results for DNA purified by sequence capture and adsorption to silica). It is noteworthy that for six of these patients, mycobacteria were never isolated by culture from any specimen submitted. For three of these culture-negative patients, pleural fluid samples gave positive results by sequence capture-PCR; these findings represented the only direct evidence for the presence of *M. tuberculosis* in specimens from these patients.

For each sample from a patient with tuberculosis, three or four control samples were processed in parallel during all steps of the procedure (solubilization of samples, purification of mycobacterial DNA by sequence capture, and amplification). Two fragments of a rat spleen were evaluated to ensure that reagents were not contaminated with mycobacterial DNA and that no transfer of mycobacterial DNA occurred during processing. In addition, one ($n = 2$) or two ($n = 15$) samples of pleural fluid from patients without tuberculosis were tested to evaluate the possibility that mycobacterial DNA could be recovered from individuals without active tuberculosis. None of these control samples gave positive results (0 of 34 animal tissue and 0 of 32 nontuberculous pleural fluid samples).

Amplification of the DR sequence from the *M. tuberculosis* complex by sequence capture-PCR. Sequences present in multiple copies in the *M. tuberculosis* genome are particularly attractive targets for sequence capture. Although most strains of *M. tuberculosis* contain multiple copies of IS*6110*, some strains have few copies; in certain geographical areas, strains not containing IS*6110* are prevalent (27). Therefore, we also developed a sequence capture technique that targets an alternative mycobacterial sequence, the DR sequence. This sequence, which is also specific for the *M. tuberculosis* complex, is present as multiple highly conserved tandem repeats of 36 bp, each separated by a 35- to 41-bp spacer sequence (14). Unlike the DRs, each of these spacers has a unique sequence. Oligonucleotides DRa and DRb, which amplify fragments of variable lengths between two different DR sequences (including the intervening spacer and DR sequences), were used to amplify this region (16).

When samples containing known amounts of purified mycobacterial DNA in 500 ng of human DNA were amplified, positive results were obtained for 11 of 11 samples containing 2 to 10 mycobacterial genomes, 17 of 28 samples containing 1 genome, and 0 of 9 samples containing 0.1 genome. The lower-level sensitivity of the DR system compared with that of the IS*6110* system for the detection of purified mycobacterial DNA is expected. Unlike the IS*6110* sequence, which is dispersed in multiple copies throughout the mycobacterial genome of the mycobacterial strain used as a standard in these studies, the repeated DR sequences are present at a single locus and therefore are likely to be present on a single DNA fragment. Thus, at limiting dilutions (≤1 genome per sample), individual aliquots are less likely to contain fragments with the DR sequence than fragments containing the IS*6110* sequence.

When the sequence capture-PCR protocol was used, however, marked differences in sensitivity between the DR and

TABLE 2. Comparison of the detection of mycobacteria in clinical samples by standard bacteriological techniques and amplification of mycobacterial DNA

Patients[a]	No. of positive sputum samples/no. tested		Result with pleural fluid[b]			
			Bacteriology		Amplification of IS*6110*	
	Acid-fast stain	Culture	Acid-fast stain	Culture	Silica adsorption	Sequence capture
1	0/3	3/3	−	+	+	+
2	0/3	0/3	−	+	+	+
3	0/2	0/2	−	+	+	+
4	0/3	1/3	−	−	−	+
5	0/3	2/3	−	−	−	+
6‡	0/3	0/3	−	−	−	+
7	3/3	3/3	−	−	−	+
8‡	0/3	0/3	−	−	−	+
9	0/3	1/3	−	−	−	+
10	3/3	3/3	−	−	−	+
11‡	0/3	0/3	−	−	−	+
12	0/3	1/3	−	−	−	+
13	0/3	3/3	−	−	ND	+
14‡	0/3	0/3	−	−	−	−
15†	0/3	0/3	−	−	−	−
16‡	0/3	0/3	−	−	−	−
17‡	0/3	0/3	−	−	−	−

[a] †, patient for whom culture of pleural biopsy was positive; ‡, patient for whom all cultures submitted were negative for mycobacteria.
[b] +, positive result; −, negative result; ND, not done.

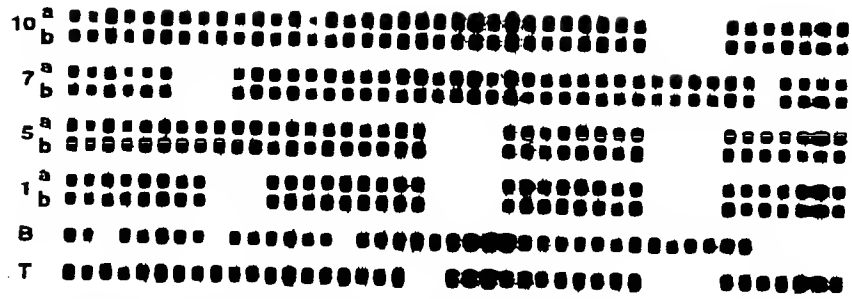

FIG. 3. Typing of mycobacterial strains by spoligotyping after sequence capture-PCR. Sequence capture-PCR targeting the DR region was performed as outlined in Fig. 1 on pleural fluid samples from patients with tuberculous pleurisy. For samples from individuals for which two independent reactions gave positive results, aliquots of the amplification products were reamplified by the DRa-DRb primer pair in which the DRa oligonucleotide was biotinylated. The amplification products were then hybridized to membranes to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *M. bovis* BCG had been covalently linked, and positive hybridization reactions were identified by detecting the presence of biotinylated amplification products using the ECL detection system (Boehringer Mannheim). Spacer oligonucleotides are displayed in numerical order from left to right on the membrane. Shown are the results for four of the five patients (patients 1, 5, 7, 8, and 10) for which the spoligotyping profiles from two independent reactions (a and b) were identical. Note that the profiles are unique for each patient and distinct from those obtained with DNAs from *M. tuberculosis* H37Rv (T) and *M. bovis* BCG (B).

IS*6110* systems were not observed. First, sequence capture-PCR targeting the DR sequence was performed on samples containing small amounts of mycobacterial DNA added to crude extracts of animal tissues or human immune and inflammatory cells containing up to 750 μg of human DNA. Positive results were obtained for 25 of 26 samples containing 10 mycobacterial genomes and 2 of 6 samples containing 1 mycobacterial genome, results not significantly different from those obtained by using the IS*6110* system (Table 1). Similarly, crude extracts of DNA recovered from pleural fluid samples of patients with tuberculous pleurisy, available from 15 patients evaluated by using the IS*6110* system, were also tested by the DR sequence capture technique. Positive results were obtained for 10 of 15 specimens, including all three samples that were culture positive. For 12 samples, the results were concordant between the two systems, although 2 samples positive by using the IS*6110* system were negative by using the DR system and 1 sample positive by using the DR system was negative by using the IS*6110* system.

Typing of mycobacterial strains after sequence capture-PCR. Although all strains of *M. tuberculosis* contain the DR sequence, the spacer sequences present are different for different strains. Kamerbeek et al. (16) have used this observation to develop a technique to type mycobacterial strains on the basis of the hybridization of amplification products of the DR region to a panel of synthetic oligonucleotides specifically recognizing different spacer sequences (spoligotyping). To determine whether the amplification products obtained from the pleural fluid samples of patients with tuberculous pleurisy were adequate to permit rapid typing, this approach was applied to these samples.

It has previously been shown that when extremely small amounts of mycobacterial DNA are used, amplification of only a portion of the DR region may occur, producing incomplete spoligotyping profiles. Although this is not a problem when DNA is extracted from cultured mycobacteria, it is a potential problem when spoligotyping is applied to mycobacterial DNA obtained from paucibacillary clinical samples such as those studied here. To guard against this possibility, typing was restricted to samples for which positive results were obtained for both of the independent amplification reactions and for which the spoligotyping profiles were identical for two independent reactions. These criteria were met for 5 of the 10 pleural fluid samples that were positive for mycobacterial DNA after amplification of the DR region, and the spoligotyping profiles are shown in Fig. 3. In each case, the profiles were distinct and different from that of *M. tuberculosis* H37Rv, the strain used as a positive control in these experiments. Thus, none of the patients was infected with the same mycobacterial strain, and in no case could positive results be explained by the inadvertent contamination of the sample with DNA from another patient or the control strain.

DISCUSSION

In this study, we have developed a new PCR-based strategy, sequence capture-PCR, that permits the rapid enrichment of mycobacterial DNA present in crude extracts of clinical samples prior to amplification and thereby results in a substantial increase in sensitivity of detection of mycobacterial DNA in these specimens. By using samples containing known amounts of DNA, this approach was shown to be 10 to 100 times more sensitive than are procedures in which total DNA is extracted prior to amplification. Furthermore, this improved sensitivity was shown to result in a much higher proportion of positive results when clinical samples from patients with tuberculous pleurisy were tested; only sequence capture-PCR permitted the detection and typing of mycobacteria in a majority of culture-negative specimens from patients with tuberculosis.

The specific capture of nucleic acids by immobilized oligonucleotides has numerous applications in molecular biology but has not found wide application in diagnostic tests. Muir et al. (20) used oligonucleotides coupled to magnetic beads to capture enteroviral RNA prior to reverse transcription-PCR. They found that although this method was simpler to perform, the sensitivity was similar to that obtained by traditional extraction techniques. We found, however, that when oligonucleotides recognizing mycobacterial DNA were directly coupled to beads (direct capture), the efficiency of capture of mycobacterial DNA was much less than that when the biotinylated oligonucleotides were hybridized to mycobacterial DNA in solution and subsequently bound to avidin-coated beads (two-step capture). The reasons that direct capture was less efficient were not studied, but it may result from poor diffusion of the immobilized oligonucleotides and/or steric interference by the large beads. In practice, two-step capture was no more difficult to perform; the only disadvantage is the risk that endogenous biotin could impair efficient binding of biotinylated oligonucleotides. Endogenous biotin was not found in clinical specimens of lungs, lymph nodes, pleural fluids, or

peripheral blood leukocytes. When present (e.g., biopsies of livers and kidneys), it could be removed by pretreating samples with avidin-Sepharose prior to capture (unpublished data).

Our study confirms prior reports (18) that the sensitivity of detection of rare target sequences by PCR is highly dependent on the amount of total DNA in the sample; the sensitivity of detection of mycobacterial DNA was clearly lower in samples containing more than 1 to 2 μg of total DNA in a 100-μl reaction mixture. Because many clinical samples, such as the pleural fluid samples studied here, contain several milligrams of DNA, only a small fraction of the sample can be used when total DNA is studied. In contrast, sequence capture-PCR eliminates essentially all cellular DNA, thereby permitting the analysis of all or the majority of the sample in a single reaction. We have demonstrated that mycobacterial DNA can be detected in a variety of clinical samples, including samples containing large amounts of DNA (e.g., sputum, tissue biopsies, and peripheral blood cells). In addition, sequence capture eliminates potential inhibitory substances present in crude samples. For example, we found that mycobacterial DNA present in tissues containing large amounts of hemoglobin or those extracted with 1% sodium dodecyl sulfate, both strong inhibitors of *Taq* polymerase, could be successfully amplified after sequence capture.

An important finding in the present study was the observation that sequence capture-PCR permitted the detection of mycobacterial DNA in the majority of culture-negative pleural fluid samples from patients with tuberculosis. Prior studies have reported detecting mycobacterial DNA in culture-negative specimens from patients with tuberculosis (7, 11–13, 19, 22, 24), indicating that nonviable organisms can be present in these samples because of the mycobactericidal action of inflammatory cells or loss of viability attendant with sample processing. Nevertheless, in previous studies by us and other groups in which total DNA was amplified, only occasional culture-negative samples gave reproducibly positive results. In contrast, sequence capture-PCR gave positive results for 10 of 14 culture-negative samples. For three of the patients studied here, the detection of mycobacterial DNA by sequence capture-PCR was the only direct evidence for the presence of *M. tuberculosis* in these patients, as multiple sputum, pleural fluid, and pleural biopsy cultures were negative.

Systems permitting the amplification of two different mycobacterial sequences, IS*6110* and the DR region, were developed in these studies. Both were shown to be highly efficient in detecting DNA from as few as 10 mycobacteria in 750 μg of total DNA, and the sensitivities of these two systems for the detection of mycobacterial DNA in tuberculous effusions were not different. These results suggest that sequence capture-PCR can be applied to a variety of different target sequences. Further studies will be needed to rigorously compare the sensitivities of the two systems described here in clinical practice, but two potential advantages of the DR system merit mention. First, the DR sequence is always present in organisms of the *M. tuberculosis* complex in multiple copies; strains not containing this sequence have not been identified. In contrast, the IS*6110* sequence is present in only one or two copies in many *M. tuberculosis* strains and strains lacking IS*6110* have been reported (1, 26, 27). Second, as confirmed in this study, amplification products generated by amplifying the DR region can be used to type the mycobacterial strain detected, thereby permitting rapid identification of community outbreaks or nosocomial infection. Current work in our laboratory is directed at automating the sequence capture-PCR procedure, thereby permitting routine clinical use of this highly sensitive approach.

ACKNOWLEDGMENTS

These studies were supported in part by grants from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés (CNAMTS) and the Fondation pour la Recherche Médicale.

We thank Annelies Bunschoten for excellent technical assistance and Veronique Vincent-Lévy-Frébault for providing the standard mycobacterial DNA.

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CLINICAL MICROBIOLOGY REVIEWS, Apr. 1997, p. 242–256
0893-8512/97/$04.00+0

Vol. 10, No. 2

Relevance of Nucleic Acid Amplification Techniques for Diagnosis of Respiratory Tract Infections in the Clinical Laboratory

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INTRODUCTION

During the last 5 to 7 years, the advantages of diagnostic molecular techniques have been so widely publicized that increasing pressure has been placed on clinical microbiology laboratories to apply them for the detection of a wide variety of infectious agents, especially since test kits for some applications are being made commercially available. In this paper, we review the efficiency and practicability of nucleic acid amplification techniques for the diagnosis of respiratory tract infections.

Before introducing molecular techniques in the diagnostic laboratory, several strategic questions must be addressed: which organisms should be targeted; which clinical specimens should be tested; and do these molecular tests fulfill the required criteria of high sensitivity and specificity, speed, simplicity, and clinical relevance? In general, molecular diagnostic techniques are indicated (i) for the detection of organisms that cannot be grown in vitro or for which current culture techniques are too insensitive, or (ii) for the detection of organisms requiring complex media or cell cultures and/or prolonged incubation times. For respiratory infections, the following organisms meet the criteria described above: rhinoviruses, coronaviruses, hantaviruses, *Bordetella pertussis*, *Legionella* species, *Coxiella burnetii*, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, fungi, and *Pneumocystis carinii*.

This review concentrates on those respiratory agents for which considerable numbers of clinical specimens have been examined. Studies concerning the development of tests for the corresponding pathogens are not considered. Respiratory disease due to cytomegalovirus is not discussed because it does not result from an airborne infection but most frequently from a reactivation of a latent infection in relation to an immunosuppressive state, in which the interpretation of the virological investigations poses particular problems.

The basic principle of any molecular diagnostic test is the detection of a specific nucleic acid sequence by hybridization to a complementary sequence, a probe, followed by detection of the hybrid (21). However, the sensitivity of nucleic acid probe tests that do not involve amplification is lower than that of classical diagnostic tests (191). This lack of sensitivity applies to the detection of respiratory pathogens including rhinoviruses (3, 16), *M. pneumoniae* (71, 102, 103, 176), *C. pneumoniae* (19), and *M. tuberculosis* (150). The main use of the nonamplification probe procedure is in the identification rather than the detection of microorganisms (32, 45).

Thereupon, techniques have been developed to amplify the target nucleic acid or the probe. Any stretch of nucleic acid can be copied by using DNA polymerase, provided that some sequence data are known to allow the design of appropriate primers. DNA replication was made possible in 1958, when Kornberg discovered the DNA polymerase (106). For many years, one of the main applications of this discovery was in the DNA-sequencing procedure of Sanger et al. (166). In 1986, Mullis et al. (132) introduced a reiterative process, PCR, which leads to an exponential increase in the production of the nucleic acid. In view of the immense number of possible appli-

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cations in the most diverse fields, commercial interest was immediately awakened. Alternative nucleic acid amplification techniques were developed and patented, using different enzymes and strategies, but they are all based on reiterative reactions (29, 60, 110, 115, 216).

Nucleic acid amplification techniques can be classified by several criteria. Conceptually, there are those in which the target nucleic acid is amplified and those in which the probe is multiplied (21, 215); from a practical point of view, there are the in-house-developed applications and the commercially available tests. Target nucleic acid amplification techniques include PCR, the strand displacement amplification, and the isothermal RNA self-sustaining sequence replication reaction, from which the commercialized nucleic acid sequence-based amplification (NASBA) and the transcription-mediated amplification (TMA) are derived. The ligase chain reaction (LCR), in the so-called gapped LCR format, is a combination of target and probe amplification. The Qβ replicase amplification (QβRA) involves probe amplification only.

PCR (132) consists of a number of temperature cycles, each cycle consisting of two or three temperature steps: denaturation to ensure the separation of the target DNA duplexes, annealing to allow added synthetic oligonucleotide primers to hybridize to the DNA target, and extension to allow the added DNA polymerase to synthesize complementary DNA strands. In some protocols, annealing and extension occur at the same temperature. After a series of these temperature cycles, the specific PCR product or amplicon, consisting of the two primers bridged by the intervening nucleotide sequence, accumulates. Modifications of the basic procedure are nested PCR (149), multiplex PCR (25), and reverse transcriptase (RT) PCR (149).

In a nested PCR (149), a second round of amplification is performed, using the amplicon of the first round as a target and a pair of primers complementary to sequences within this amplicon, the amplicon of the second reaction being shorter than that of the first. The advantage of nested PCR is increased sensitivity, but this is achieved at the cost of a high risk of cross-contamination, since the tubes containing amplicons have to be opened after the first stage to add new reagents for the second stage. It also increases the specificity of the reaction since the internal primers anneal only if the amplicon has the corresponding, expected, sequence.

In a multiplex PCR (25) several independent amplifications are carried out simultaneously in one tube with a mixture of primers. However, since the annealing temperatures for the respective primer pairs are not necessarily identical, problems of specificity of the individual reactions may result.

In an RT-PCR (149), an RNA target, usually viral RNA, is first transcribed into complementary DNA, enabling the PCR to proceed.

The TMA and NASBA (29, 60) amplify RNA via the simultaneous action of three enzymes: an RT (which also has polymerase activity), an RNase, and an RNA polymerase. The synthesis of cDNA is primed by specially designed oligonucleotide primers, one end of which is a target-specific sequence, while the other end contains a promoter for the RNA polymerase. The RT synthesizes an RNA-DNA hybrid, the RNase digests the RNA component, and the RT synthesizes double-stranded DNA; finally, the RNA polymerase produces numerous RNA copies.

In the LCR (216), after heat denaturation of the double-stranded DNA, two pairs of primers anneal to each strand of the target. A DNA ligase joins the primers, and the ligation product is released by heating and serves as template for new ligations. In the gapped LCR (110), a gap of 1 to 3 bases is left between the primers and is filled in by the action of added DNA polymerase, before the primers are covalently linked by a ligase. In subsequent cycles, the ligated primers act as targets for further annealing and ligation.

In the QβRA (115), a specifically synthesized RNA probe is used. It contains a sequence specific for a target, either DNA or RNA, a sequence to enable the capture of the probe-template hybrids, and a sequence recognized by the Qβ replicase enzyme to start replication. After annealing of the probe to the target, the hybrids are captured, and the probe is removed enzymatically and amplified by the Qβ replicase. This technique is still largely in the developmental stage, the main difficulty being the separation of nonhybridized from hybridized probe before amplification.

Each of the amplification techniques is composed of three parts: sample preparation, amplification, and product detection. The sample preparation step involves primarily the liberation and concentration of the target nucleic acid and the elimination of amplification inhibitors. A great diversity of sample preparation procedures has been described, particularly for PCR. Inhibitors occur frequently and may be difficult to eliminate: heme compounds (79) and polysaccharides in sputum (109), as well as some reagents (67) and components of swabs (207).

The amplification step should aim at maximum sensitivity and specificity through judicious choice of the primers and optimal temperatures when thermocyling is involved, offer maximum protection against contamination, and include proper positive and negative controls. The purpose of the positive control is to monitor the amplification process, particularly to detect inhibitors of the reaction. Concomitant amplification of human β-globin has been used frequently for this purpose. At the same time, it determines the presence of host cell material, which is particularly useful after elaborate sample preparation procedures. However, it requires the introduction of specific primers into the reaction, resulting in a multiplex PCR. To avoid this problem, a PCR for the globin is sometimes performed in a separate tube, but the optimal cycling temperatures for this internal control may differ from those required for the principal reaction. Therefore, specific, positive internal controls are preferred. These are modified amplicons that have been made shorter or longer and are added to each reaction tube. Their ends are identical to those of the target, and therefore they are amplified by the same reagents as the real target, but they are easily differentiated from it by being shorter or longer (6, 38, 44, 64, 96, 105, 137, 148, 151, 167, 188, 197, 198). By adding specific positive internal controls to the samples at the very start of the process, the efficacy of the sample preparation procedure can be assessed. Moreover, the addition of specific internal controls avoids the use of reference organisms or their nucleic acid as positive, external controls, thus eliminating an important possible source of contamination. The addition of a limited amount of internal control should not significantly reduce the sensitivity of the procedure, and it offers greater advantages than disadvantages (198). Internal controls allow also the quantitation of the reaction (96).

Negative controls are target-free samples, usually distilled water, which are subjected to the same manipulations as the test samples. Their purpose is to detect contaminations between reaction tubes. Indeed, after numerous exponential nucleic acid amplifications, there are ample sources of cross-contamination in the laboratory. The greater the number of manipulations, the greater the risk of cross-contamination among the specimens, especially if multiple centrifugations are required. Appropriate measures should be taken to avoid con-

TABLE 1. Diagnostic methods for respiratory viruses

Etiologic agent	Rapid conventional methods available	PCR	
		Relevant	Reference(s)
Adenoviruses	+	No	
Influenza viruses	+	No	27
Parainfluenza viruses	+	No	
RSV	+	No	146
Herpes simplex virus	+	No	
Rhinoviruses	−	Yes	7, 69, 89, 91
Coronaviruses	−	Yes	133
Enteroviruses	−	Yes	91

tamination. These measures include the use of three different rooms with restricted access for each of the reaction steps, the use of appropriate pipette tips, and cleaning of the area by UV irradiation, or the use in the PCR of dUTP instead of dTTP, allowing disintegration of unwanted, possibly contaminating, amplicons by uracil-*N'*-glycosylase (177).

Because of the exquisite sensitivity of nucleic acid amplification tests, there should be a constant awareness of the possibility of false-positive results. These not only are due to cross-contaminations in the laboratory but also may result from contaminations during sampling, particularly when organisms, such as fungi or legionellas present in the environment, are studied. Samples from treated patients may remain positive for prolonged periods (39, 63, 75). For all these reasons, confirmation of the existence of some microorganisms in subclinical infections or a carrier state becomes difficult.

In the PCR and the LCR, the amplicons can be detected by gel electrophoresis, followed or not by solid- or liquid-phase hybridization with a specific probe, by fluorescence (88), or by an enzyme immunoassay (EIA) reaction. Hybridization can increase the sensitivity of the detection 10- or 100-fold. The amplicons of NASBA and TMA are detected by hybridization or by a commercial luminescence reaction (41), and those of the QβRA can be detected by an incorporated fluorescent dye.

At present, PCR is undoubtedly the most widely used amplification technique, probably because it was the first one described and was introduced rapidly in innumerable laboratories for a wide variety of applications. Commercial formats of PCR (Roche), TMA (MTDT, GenProbe), NASBA (Organon Teknika), and LCR (Abbott) have been developed, particularly for infectious agents for which large numbers of clinical specimens are tested: sexually transmitted agents (*Neisseria gonorrhoeae*, *C. trachomatis*, human immunodeficiency viruses), hepatitis C virus, and *M. tuberculosis*. In these formats, the amplicon is detected either by a semiautomated EIA reaction (Roche) or by an electrochemiluminescence procedure or a hybridization reaction (Organon Teknika), or it is coupled to an existing acridinium ester luminescent nucleic acid probe technique (GenProbe) or a previously developed, automated EIA technique (Abbott).

In-house tests are more versatile and can easily be applied to any target by switching to the appropriate primers and, if necessary, adapting the cycling temperatures accordingly.

MOLECULAR DIAGNOSTIC TECHNIQUES FOR ACUTE RESPIRATORY TRACT INFECTIONS

Viruses

Table 1 illustrates the present situation for the diagnosis of adenovirus, influenza virus, parainfluenza virus, and respiratory syncytial virus (RSV) infections for which rapid conventional techniques are available: influenza virus and RSV can be detected in the clinical specimens by immunofluorescence and parainfluenza virus and adenovirus can be detected by immunofluorescence after incubation for 48 h in shell vial cultures (147). In these cases, nucleic acid amplification techniques have no added value in terms of sensitivity or rapidity. In one study (27), comparing PCR with conventional techniques for the detection of influenza virus, the authors concluded that there are no arguments for the introduction of PCR for the diagnosis of influenza virus infection. In a study by Paton et al. (146), PCR for RSV had a sensitivity of 94.6% and a specificity of 97%; the molecular technique detected 1% of cases undiagnosed by culture and EIA. Clearly, PCR does not represent significant improvement over existing methods for the detection of these viruses.

Rhinoviruses and coronaviruses grow poorly in cell culture. In addition, rapid immunofluorescence and/or culture techniques are not available for the direct detection of these viruses in clinical specimens (7, 69). Typically, rhinoviruses are isolated in roller cultures, sometimes after several blind passages, followed by acid lability testing. More than 100 serotypes are known. PCR is much more sensitive than is culture (136): Ireland et al. (89) and Johnston et al. (91) detected five and three times as many rhinoviruses by PCR, respectively, compared with the best available cell culture techniques. In another study (59), significantly more multiple-virus infections by RSV, parainfluenza viruses, and rhinoviruses were detected by RT-PCR than by culture. However, some technical details must still be worked out. To detect the large number of rhinovirus serotypes, regions within the conserved noncoding 5′ untranslated region of the genome are amplified (54), leading to cross-reactions with many enteroviruses. Several methods have been used to detect rhinoviruses specifically: a nested procedure, the use of primers spanning a region between the 5′ untranslated region and the VP2/VP4 region, hybridization with specific probes (69), and differentiation on the basis of the size of the amplicons (89, 141, 196) or sequencing (131). Nevertheless, Johnston et al. (91) could identify only 8 of 30 positive samples as rhinoviruses on the basis of either acid lability or the length of the amplicon, with 73% remaining "unclassified picornaviruses." Another problem emerging from studies on human rhinoviruses by PCR is whether healthy carriers exist: 12 and 4% of samples from asymptomatic children and adults, respectively, were positive for picornavirus by PCR (91).

Clearly, there is still more to learn about the epidemiology of rhinoviruses, particularly in children, infants, and the elderly. Molecular diagnostic techniques offer the necessary tools.

A PCR based on the genomic sequences of the two known human coronavirus strains, 229E and OC43, is available (133), and it is highly likely that more, as yet uncultivated, human coronaviruses remain to be detected. No extensive studies to define better the role of coronaviruses in respiratory infections have been undertaken.

Hantavirus pulmonary syndrome, a rodent-borne infection, appeared in 1993 and 1994 in the New Mexico-Arizona-Colorado area. It is characterized by fever, myalgias, headache, and cough, followed rapidly by respiratory failure. Antibodies against heterologous hantavirus antigens were initially used to identify the causative agent, and then the hantavirus genome was detected by PCR in autopsy specimens (135). Specific genetic recombinant-derived proteins were prepared from viral genomic sequences amplified from tissues obtained from patients who died of confirmed hantavirus illness (108). Since the virus has not yet been cultured, PCR with specific primers and serology are the only diagnostic possibilities.

Rapid diagnostic techniques for respiratory pathogens are not only important for clinical-epidemiological reasons but are also useful so that treatment can be appropriately initiated within the first 24 h or halted when the symptoms are found to be caused by another microbial agent.

Bacteria

Bordetella pertussis. Despite the routine immunization of children, pertussis continues to be an important disease in infants and young children. During the last 2 years, there has been a resurgence of pertussis in the United States (24), Italy, the Russian Federation, and Sweden (165). In 1994, approximately 3,500 to 4,000 cases were reported to the Centers for Disease Control and Prevention in the United States (24). These figures probably underestimate the true incidence of pertussis because of the difficulty in confirming the diagnosis (70, 182). The major reservoir for pertussis now appears to be previously vaccinated adolescents and adults with atypical and often unrecognized symptoms of pertussis. Making the clinical diagnosis of pertussis in this reservoir is more challenging because many of these patients do not have the classic coughing paroxysms or "whoops."

The conventional laboratory diagnosis of pertussis has relied on culture, direct immunofluorescence, and serologic testing. Each of these methods has problems with either sensitivity or specificity (47, 70, 182). Diagnosis by culture is specific but not very sensitive since most individuals are culture negative at the time when clinical symptoms are apparent. Direct immunofluorescence is prone to a large number of false-positive results, and when used on a single specimen, serologic testing is often nonspecific. Follow-up confirmation with a second specimen would result in a 3- to 4-week delay in the diagnosis. These problems have led to an inability to confirm the diagnosis in many patients, and therefore nucleic acid amplification techniques, in practice PCR, have been used (8, 40, 47, 68, 72, 73, 169, 170, 199). The presence of a repetitive gene element in *B. pertussis* increases the sensitivity of the PCR. The reaction allows also a clear-cut distinction between the pathogenic *B. pertussis* and the usually nonpathogenic *B. parapertussis* (199).

An unexpected origin of false-positive PCR results for *B. pertussis* was described by Taranger et al. (187). Pharyngeal samples were obtained in a room that was grossly contaminated with pertussis DNA because killed, whole-cell pertussis vaccine was administered in the same room.

In a recent report (123), several aspects of PCR-based detection of *B. pertussis* were discussed. The main conclusions, which we can support, were that (i) there are no comparative studies between the different PCR procedures; (ii) although the PCR procedures used in different laboratories can detect 80 to 100% of the culture-positive samples, the percentage of PCR-positive samples that were culture negative differed by 13 to 88%; (iii) there is need for rigorous control of false-positive and false-negative results; (iv) questionable results must be confirmed by a second method; and (v) PCR-positive results are acceptable only for individuals with classical symptoms of pertussis. The clinical and epidemiological significance of a PCR-positive result in someone with mild or no symptoms should be interpreted with caution, and, if possible, other markers, such as serologic tests or epidemiologic data should be used in addition. Finally, it is too early to recommend a standard PCR technique for the detection of *B. pertussis* in clinical specimens, because no comparative studies have been done.

***Legionella* species.** Legionellae are ubiquitously distributed in natural and man-made water systems (49, 206). Respiratory infections caused by *Legionella* spp. often occur in immunodeficient persons. Cultures of bronchoalveolar lavage specimens take a minimum of 48 to 72 h to grow, and plates should be incubated for 7 days. Jaulhac et al. (90) applied PCR retrospectively to frozen bronchoalveolar lavage specimens. They confirmed all culture-positive specimens and found additional specimens positive by PCR from patients whose clinical features were in accordance with legionellosis. Kessler et al. (98), in a prospective study combining a rapid DNA extraction procedure with a commercial kit for the amplification and detection of legionellae in environmental samples, detected the organisms in all specimens later confirmed by culture. In another study (125), legionellae were detected by PCR but not by conventional culture.

In an effort to detect *Legionella* infections by the examination of specimens obtained by less invasive procedures, Maiwald et al. (120) examined urine specimens from experimentally infected guinea pigs and patients by an EIA and by PCR. PCR was more sensitive than EIA in detecting legionellae, and two urine samples were intermittently positive, indicating that DNA is not continuously excreted. The advantage of PCR over EIA is that PCR is a genus-specific reaction whereas antigen detection must be performed with a variety of serogroup reagents to cover the spectrum of possible causative species. The authors concluded that a more detailed prospective study of hospitalized patients with pneumonia is warranted. Their results also illustrate the recurring problem of contamination associated with amplification techniques, since 3 of 30 control samples from patients with urinary tract infections were positive, possibly as a result of contamination by hospital water.

The need for nucleic acid amplification techniques for *Legionella* infections can be questioned in view of their relatively easy isolation from respiratory specimens within a moderate time span and the ability to prevent nosocomial legionellosis by control of legionellae in the hospital plumbing system (114). PCR may be more suitable for the detection of legionellae in environmental specimens to avoid overgrowth by contaminating organisms (119).

Coxiella burnetii. *C. burnetii* is a fastidious intracellular bacterium. Different strains show heterogeneity in their growth conditions, with some being very difficult to culture in vitro. The isolation of *C. burnetii* was greatly improved and facilitated by application of the shell vial assay technique (159), which produced results within 6 days. A PCR for *C. burnetii* (181) has been shown to be very sensitive and specific and is able to produce results within 6 h. It can be applied to inoculated shell vials or directly to clinical specimens. For the time being, this procedure will remain restricted to reference laboratories in countries or areas where the disease does occur, as illustrated recently by To et al. (194).

***Chlamydia* species.** Three *Chlamydia* species are responsible for human respiratory infections: *C. psittaci* and *C. pneumoniae* in adults and older children, and *C. trachomatis* in newborns, who are infected during delivery.

The last organism has been implicated, by serology (2), in 3 to 18% of all cases of infant pneumonitis. Although nucleic acid amplification techniques for the detection of *C. trachomatis* in genitourinary specimens have been intensively studied, there are no such studies on respiratory specimens. It could well be that the techniques used for genitourinary specimens cannot be applied unchanged to respiratory specimens, particularly the specimen preparation procedure (41).

C. psittaci may be an important human pathogen in some areas and may be underdiagnosed on the basis of serologic testing alone. Since respiratory infections by *C. trachomatis* and *C. psittaci* occur sporadically, there has been less need or

opportunity for the application of amplification techniques for these infections. Several research groups have developed a two-step procedure for the successive detection of organisms belonging to this genus and their subsequent identification to the species level, by the amplification of a common genus-specific DNA sequence followed by digestion with restriction enzymes (80, 160, 210) or by a nested PCR (195). None of these procedures has been applied on a significant scale.

The role of *C. pneumoniae* in disease was discovered relatively recently, but the insensitivity of cell culture techniques has hampered extensive clinical and epidemiological investigations. In addition, serologic tests are labor-intensive, since they rely on microimmunofluorescence tests for detection of both immunoglobulin M (IgM) and IgG. Serologic investigations seem to indicate that the culture technique fails to detect many infections. However, taking into account the shortcomings of serologic testing, in terms of specificity and sensitivity (58), it can be surmised that the techniques available fail to diagnose *C. pneumoniae* infections to an unknown extent, although the organism does not seem to be a common cause of respiratory infection in children (65). Therefore, several PCR primer sets have been developed to detect either outer membrane or 16S rRNA coding genes (10, 19, 55, 58, 66, 143, 157, 160).

One of the difficulties in evaluating nucleic acid amplification tests for the diagnosis of *C. pneumoniae* infections is the choice of the reference or "gold standard." Because culture is relatively insensitive, many studies refer to serologic results, considering the presence of IgM, a fourfold increase in antibody titers during and after the acute disease episode, or an IgG titer of at least 512 to be significant. The presence of clinical symptoms cannot be taken into account, since asymptomatic infections by *C. pneumoniae* have been documented by culture and PCR (84).

In addition to this problem of the appropriate reference method to use for the detection of *C. pneumoniae*, inhibitors of PCR are common components of the specimens. Some solutions have been proposed, including the use of samples such as gargled water, throat swabs, or nasopharyngeal swabs instead of nasopharyngeal aspirates or sputum (157, 195), alternative sample treatment methods (62, 117), and introduction of a nested PCR (11).

In all studies in which they were compared, PCR detected 10 to 20% more cases than culture, but in turn serologic determination detected 10 to 20% more cases than PCR. In one study (58), when compared with the combination of a positive culture and direct immunofluorescence test, the PCR had a sensitivity of 76.5% and a specificity of 99%; when compared with the combination of a positive PCR and direct immunofluorescence test, the sensitivity of culture was 87.5%. In the same study, only 8 acute-phase serum specimens (23%) of the 35 *C. pneumoniae* culture- or PCR-positive patients had a diagnostic antibody titer, as did 18.8% of those from 80 asymptomatic persons. Thorn et al. (192) diagnosed 21 cases by serologic testing among 743 middle-aged and older patients; 15 of the patients were positive by PCR. Gaydos et al. (56) studied 132 *C. pneumoniae* culture-negative BAL specimens from 108 immunocompromised patients. A total of 20 *C. pneumoniae* infections were diagnosed: 8 by PCR, 4 by PCR and serologic testing, and 8 by serologic testing alone. In this study, PCR and serologic testing had a sensitivity and specificity of 33.3 and 91%, respectively, and both detected 60% of the cases. Thus, it seems that both conventional culture and PCR diagnose only a fraction of the total number of cases and that the diagnosis of individual infections by serology is by no means straightforward, due to the occurrence of many false-negative and false-positive results.

Many aspects of the diagnosis of *C. pneumoniae* infections by amplification techniques remain to be explored. There is need for an internal control; for comparisons of different types of samples, sample preparation methods, and primers; and for several amplification techniques to be performed on the same specimens.

Mycoplasma pneumoniae. *M. pneumoniae* grows slowly in vitro, requiring 2 to 4 weeks for colonies to appear. Therefore, research laboratories have identified several genomic sequences suitable for amplification, including the P1 gene (87), the 16S rRNA gene (201), and a species-specific protein gene (116). In clinical studies, the sensitivity and specificity of amplifications based on these sequences were 90 to 94% and 97 to 100%, respectively (34, 57, 86, 94, 112, 116, 122, 176, 178, 193, 201). PCR also detected *M. pneumoniae* in specimens from 1 to 3% of healthy subjects (116, 193) or convalescent patients, raising the possibility of a carrier state or persistence of the organism in the respiratory tree. In a recent study (86), 371 nasopharyngeal aspirates from children with acute respiratory infections were examined for viruses by rapid conventional techniques and for the presence of *M. pneumoniae* by culture and several different PCR protocols in two laboratories. Each laboratory applied one sample preparation method: freezing-boiling or isothiocyanate treatment, followed by phenol-chloroform extraction. Prepared samples were exchanged between laboratories. In both laboratories, identical primers were used in the PCR directed against the P1 gene, while one laboratory also used primers against the 16S rRNA gene. A specific internal control for the P1 amplification was included (198). Samples were defined as positive if (i) culture was positive for *M. pneumoniae*, (ii) culture and PCR for the P1 and/or the 16S rRNA genes were positive, or (iii) PCR was positive for both the P1 and 16S genes after a particular extraction procedure. Samples positive by PCR for only one of the primer pairs were considered as contaminants. Compared with PCR, culture had a sensitivity of 61%. For the PCR, depending on the preparation method used, sensitivity with the P1 primers was 76.9 to 92.3% on inspection of the electrophoresis gel and 92.3% after hybridization. The specificity was 100%. Depending on the sample preparation method, amplification of the 16S rRNA gene had a sensitivity of 53.8 to 84.6% on visual inspection of the electrophoresis gel and 69.2 to 92.3% after hybridization. The specificity was 100%. It was concluded that, provided a specific positive internal control is used, sample preparation by freezing-boiling combined with PCR for the P1 gene and amplicon detection by visual inspection of the electrophoresis gel could be recommended for clinical use, although the best results were obtained by hybridization with a labeled probe. False-positive results occurred in 0.2% of the reactions. It remains to be seen whether the finding of Resnikov et al. (163) that throat swabs contain significantly fewer PCR inhibitors than do nasopharyngeal aspirates is confirmed and that the effect does not simply result from dilution.

In the same study by Ieven et al. (86), *M. pneumoniae* was found in 3.5% of the samples but significantly more often (6.9%) in those from children older than 2 years of age. *M. pneumoniae* was the third most common etiologic agent of acute respiratory infections in children, after RSV and influenza virus. In lower respiratory infections, such as bronchopneumonia and pneumonia, *M. pneumoniae* was found as frequently as RSV. PCR is unquestionably an important step forward for the diagnosis of *M. pneumoniae* infections.

Mycobacterium tuberculosis. Amplification techniques for the diagnosis of tuberculosis have attracted considerable interest, particularly with the hope of shortening the time required to detect and identify *M. tuberculosis* in respiratory specimens

TABLE 2. Evaluation of PCR for *M. tuberculosis* in different studies

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Abe et al. (1)	135	28	81.3	84.2	94.2	100	81.3	84
Beige et al. (9)	103	47	98		70		75	
Clarridge et al. (28)	>5,000	4.4	83.6	86.1	98.7	100	94.2	98.4
Forbes and Hicks (51)	734	11		85.2		97.7		83.3
Kocagöz et al. (104)	78	49		87		96		97
Miller et al. (126)	750	21	78.2	92.3				100
Miyazaki et al. (129)	323	13	97		92	100	82	100
Nolte et al. (137)	313	40	91		100		100	
Shawar et al. (175)	384	18	74	80	95	97	77	86
Yuen et al. (218)	519	8	96		85	100		

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.

such as sputum or BAL samples. It is in this field of clinical microbiology that most amplification procedures, developed both in-house and in commercialized formats, have been evaluated.

(i) Technical aspects. Many different DNA amplification targets have been proposed, such as genes encoding the 32-kDa (179), the 38-kDa (129, 219), and the 65-kDa (145, 152) antigens and the *dnaJ* (183, 184), *groEL*, and *mtb-4* genes (104, 220). Some of these are genus or group specific, with species identification requiring subsequent restriction enzyme treatment or hybridization. The target most frequently amplified is the IS*986* or IS*6110* repetitive element (43, 77), which is present at 10 to 16 copies in most *M. tuberculosis* complex isolates, thereby increasing the sensitivity of the amplification reaction. In comparative studies, tests with the IS*6110* primers were generally more sensitive and more specific than those with IS*986* (37, 76, 208). Recently, however, *M. tuberculosis* isolates without this insertion element have been discovered in Southeast Asia (33, 202, 219).

Numerous techniques for sample preparation have been proposed, including boiling; freezing-boiling; shaking with glass beads (100); sonication (17); chloroform (213), proteinase K or "chelex" (36) treatments and combinations of these treatments; resin treatment (4); and more complex nucleic acid extraction methods (14). The commercial kits furnish their own sample treatment reagent.

Some PCRs are performed with dUTP instead of dTTP, allowing decontamination with uracil-*N'*-glycosylase (217). Both single and nested PCR formats (129, 152, 176, 213) have been applied, sometimes with the explicit purpose of overcoming PCR inhibitors.

Internal controls have been used (6, 38, 44, 105, 137, 139). However, they were only occasionally added to the specimens before the DNA extraction procedure, as was done by Kolk et al. (105). By being present during the entire procedure, an internal control not only detects inhibitors but also monitors the efficacy of the sample preparation method. Inhibitors have been detected in 3.7 to 16% of clinical samples (28, 51, 139). Curiously, Nolte et al. (137) detected inhibitors in 17% of the samples with β-globin primers but only in 10% with a specific internal control.

(ii) Results on sputum specimens with in-house PCR tests. Table 2 presents the results of nine studies in which IS*6110* was used as the amplification target. Some of these studies were done on a series of specimens with a high prevalence of positive samples. It should be remembered that for a constant rate of false-positive tests, the positive predictive value of a test decreases drastically when the prevalence of infection is low, as is the case in industrialized countries. In a population with a prevalence of <5% (in most Western European countries [139], the prevalence of positive samples is 3 to 4%), false-positive rates of 1 to 5% can lead to overdiagnosis of 50% or more of cases.

In general, the authors of the studies present their results first as "crude results", i.e., as produced by the test and thereafter as "revised results," i.e., after considering the discrepancies between the test results and the corresponding clinical information. Some authors include culture-negative, clinically diagnosed cases of tuberculosis among the "true-positives," sometimes even based on favorable response to anti-tuberculosis treatment, and thereby increase the specificity and positive predictive value of the test. None of them formulated a standard definition of a positive case except for Noordhoek et al. (139), who used the following definition of a true-positive specimen: (i) *M. tuberculosis* was cultured; or (ii) direct microscopy and PCR were positive but culture was negative; or (iii) direct microscopy and culture were negative but PCR was positive and other material from the patient was positive on culture or had been positive in the past.

None of the published studies observed a statistically significant difference between culture and the amplification technique (99). However, sensitivity and specificity are calculated as a function of the culture technique, since this is the reference method used in the absence of a better definition of a positive case of tuberculosis. In the studies, specificities vary between 85 and 100% but sensitivities are usually lower, between 74 and 97%. In one study on over 5,000 specimens (28) with a 4.4% prevalence of positive results, sensitivity, specificity, and positive predictive values were 84, 99, and 94%, respectively. By applying two primer systems in a multiplex PCR, Beige et al. (9) attained a sensitivity of 98% but a specificity of only 70%.

However, the main criticism of the use of PCR for the diagnosis of tuberculosis is a result of the separate analyses of the sensitivities of smear-positive and smear-negative, culture-positive specimens in different studies (Table 3). The test sensitivity in smear-positive cases is 88 to 100% but drops to between 50 and 92% in smear-negative cases.

One of the reasons for the lack of sensitivity may be the sample preparation method. Except for one study (139), all the procedures were applied to homogenized and decontaminated specimens as used for culture. Although this may seem appropriate when amplification techniques are compared with culture, it is not logical and may not even be optimal. In all studies

TABLE 3. Results of PCR for *M. tuberculosis* for smear-positive and smear-negative specimens

Study (reference)	PCR sensitivity (%) in different studies		
	Overall	Smear and culture positive	Smear negative, culture positive
Abe et al. (1)	84	96	50
Clarridge et al. (28)	86	94	62
Forbes and Hicks (51)	85	88	71
Miller et al. (126)	92	98	78
Nolte et al. (137)	91	95	57
Shawar et al. (175)	74	90	53
Yuen et al. (218)	96	100	92

of diagnostic amplification techniques for microorganisms other than *M. tuberculosis*, samples are divided before being allocated to the reference and amplification techniques and are thereafter prepared separately as required for each. If this were done for tuberculosis, half of the original specimen would be lysed and the nucleic acid target would be solubilized, concentrated, and introduced into the amplification reaction, thus possibly maximizing the sensitivity. In the case of paucibacillary specimens, there is a delicate balance between amplification procedures and culture. Compared with the amplification procedures, a significantly greater volume of specimen is introduced into the culture media, thus favoring the latter. However, the decontamination procedures kill 70 to 90% of the viable bacilli in the inoculum (107, 217), favoring the alternative approach. This aspect of sample preparation has been studied by Goessens et al. for the detection of *C. trachomatis* in genital specimens (63) and merits investigation for tuberculosis.

Only Noordhoek et al. (139) divided the specimens into two portions, one for conventional detection methods and one for PCR, directed at the IS*6110* element. Unfortunately, their analysis was done with a mixture of respiratory and nonrespiratory specimens, including pleural fluid, urine, and biopsy specimens. The sensitivity and specificity were 92.1 and 99.8%, respectively. PCR was negative for nine smear- and culture-positive samples. The corresponding isolates were tested and did contain the IS*6110* fragment. The authors ascribe these failures to an unequal distribution of a small number of mycobacteria present in the samples, since in each of these cases, only one or two of the three Loewenstein-Jensen culture tubes that were inoculated in parallel were positive. In this study, amplification of DNA extracted from half of the sputum specimen was not superior to culture of the other half.

In this connection, the sequence capture procedure recently described by Magiapan et al. (118) for pleural fluid specimens could be a significant advance. In this procedure, biotinylated oligonucleotides hybridize with mycobacterial DNA in the specimen and are subsequently bound to avidin-coated beads, which are introduced into the PCR mixture. Of 17 samples 13, including 3 of 3 culture-positive samples and 10 of 14 culture-negative samples, gave positive PCR results. Results of the application of this procedure to sputum specimens are eagerly awaited. The use of more appropriate primers could also enhance the sensitivity of the reaction, since even for a particular DNA sequence, different primers may result in different test sensitivities (74, 220).

Efforts to increase the sensitivity by performing a PCR on 25 μl instead of 5 μl of specimen were hampered by an unacceptable increase in the level of inhibitors (6). In contrast, by increasing the sample volume in the commercially available TMA (GenProbe MTDT) from 50 to 500 μl, one group (13) increased the sensitivity from 71.4% (obtained in a previous study [12]) to 83.3% without a loss of specificity (13).

The effectiveness of PCR for tuberculosis is related to the experience and accuracy of the personnel conducting the assay. This was illustrated by an external quality control study of seven laboratories which were tested with sputum samples spiked or not spiked with *M. tuberculosis* BCG (138). Each laboratory used its own protocol for specimen treatment and amplicon detection, but in each case the amplification target was IS*6110*. In general, false-positive rates varied between 0 and 20%, but the rate in one laboratory reached 77%; sensitivities varied between 2 and 90%. A second external quality control study of 30 laboratories, organized more recently by the same authors (140), showed no improvement: 56% of participants produced false-positive results in 5 to >50% of the samples.

(iii) Results on sputum specimens with commercially available amplification tests. The commercially available PCR (Amplicor; Roche) and TMA (Mycobacterium Tuberculosis Direct Test [MTDT]; GenProbe) test give results comparable to those obtained with in-house PCR tests (Tables 4 and 5). Sensitivities vary between 70 and 100%. The results of the MTDT for smear-positive and smear-negative specimens, respectively (Table 6), are comparable to those obtained by PCR.

Schirm et al. (168) compared an in-house PCR and the commercial PCR (Amplicor) on 504 specimens. The sensitivity of the in-house test, 92.6%, was superior to that of the Amplicor system, 70.4%, although the specificity was identical for both. More samples were inhibitory in the commercial test

TABLE 4. Evaluation of the commercially available PCR (Amplicor) for *M. tuberculosis*

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Carpentier et al. (20)	2,073	9	86		98		94.5	
D'Amato et al. (31)	985			66.7		99.7		91.7
Gleason et al. (61)	532		95		96			
Ichiyama et al. (85)	422	29	97.8		96	98.7		
Moore and Curry (130)	1,009	16	83	87	97	100		
Schirm et al. (168)	504	6	70.4		98			
Vuorinen et al. (205)	256		84.6	82.8	99.1	100		100
Wobeser et al. (214)	1,480	9.5		79		99		93

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.

TABLE 5. Evaluation of MTDT for the detection of *M. tuberculosis*

Study (reference)	No. of specimens	Prevalence (%)[a]	Sensitivity (%)		Specificity (%)		PPV (%)[b]	
			C[c]	R[c]	C	R	C	R
Abe et al. (1)	135	28	90.6	91.9	95.1	100	85.3	100
Bodmer et al. (12)	617	3	71.4		99		71.4	
Ichiyama et al. (85)	422	29	100		90.1	99.3		
Jonas et al. (92)	758	16	79.8	82.4	96.7	99.4	82	93.8
Miller et al. (126)	750	19	83.9	91	95.3	98.5	82	97
Pfyffer et al. (154)	938	8	92.9	93.9	96.2	97.6	68.4	94
Portaels et al. (156)	497[d]	4	86		96		50	80.7
	418[e]	71	97		69		89	
Vlaspolder et al. (203)	412	14	96.7	98.4	97.7	98.9	88.1	93.8
Vuorinen et al. (205)	256	13	84.6	86.2	98.7	100	100	

[a] Prevalence of positive specimens based on culture results.
[b] PPV, positive predictive value.
[c] C, crude results; R, revised results after discrepancy analysis.
[d] Belgian population.
[e] African population.

than in the in-house version. Both Ichiyama et al. (85) and Vuorinen et al. (205) compared the MTDT with the Amplicor PCR on the same specimens. In the Ichiyama study, the sensitivity and specificity of the MTDT were somewhat better than those obtained with Amplicor, but in the Vuorinen study, the results with the two test kits were similar (Tables 4 and 5).

The QβRA has been applied on a limited scale only (5, 174). The test is performed on a large volume of sputum, but the purification of the hybridized probe from the reaction mix is labor-intensive. PCR inhibitors do not interfere with the QβRA, but the procedure is very prone to amplicon contamination. In a study by Shah et al. on 261 sputum samples (174), the results were not superior to those of other amplification reactions: the sensitivity and specificity were 97.1 and 96.5%, respectively, and after revision were 97.3 and 97.8%, respectively.

Application of LCR (88) and NASBA (209) to tuberculosis has as yet been insufficiently evaluated.

(iv) **Specimens other than sputum.** PCR does not solve the problem of the bacteriological diagnosis of tuberculosis in children, who do not produce sputum. Pierre et al. (153) performed a PCR on 58 gastric aspirates, for which the classical procedures are known to have a low sensitivity. When DNA amplification was applied to two gastric aspirates from the same patient and amplified in duplicate, 25% of the specimens produced at least one positive result; when three different specimens from the same subject were examined twice, the positivity increased to 60% (in 9 of 15 children).

The diagnosis of tuberculosis by detection of *M. tuberculosis* in peripheral blood mononuclear cells, even by a molecular amplification technique, is still impractical (164), although there has been one promising study (171). The technique is more sensitive, although not optimal, in human immunodeficiency virus-infected patients, particularly in the presence of disseminated disease (50).

Since the lack of sensitivity is the main shortcoming of the amplification techniques and the specificity is more satisfactory, the tests can be useful for organism identification. When culture in a liquid medium is combined with automated growth detection and an amplification method, the time for the diagnosis of *M. tuberculosis* can be shortened to a mean of 14 days (52). PCR and MTDT assays on clinical specimens may also be useful when there is a need for rapid differentiation between *M. tuberculosis* and nontuberculous mycobacterial infections, such as in AIDS patients in industrialized countries (172).

(v) **Critique of published studies.** The published studies illustrate some shortcomings in design as well as in analysis. There should be no mixtures of respiratory and other specimens, and specimens from patients being treated should not be included. Mycobacterial DNA can be detected for a long time after the start of treatment and in the absence of positive cultures in human (75) and experimental (39) models of tuberculosis. Specimens should be divided, and each portion should be prepared independently for culture and amplification. Some patients may produce sputum with unequally distributed bacilli and/or may not excrete them continuously, and the decontamination procedures may kill variable proportions of the organisms; therefore, three specimens per patient, collected at different times or days, should be examined by each method. A definition of positivity, based on microbiological rather than clinical evidence, should be established. Culture-negative, amplification-positive specimens should be retested by an amplification reaction targeted at an alternative nucleic acid fragment to reveal false-positive results, as done by Herrera and Segovia (78). The sensitivity of the amplification method should be calculated for both the number of positive specimens and the number of positive patients.

(vi) **Conclusions concerning amplification techniques for diagnostic purposes.** At present, the conclusions published by the Centers for Disease Control and Prevention in 1993 (23) are still valid; a particular technique cannot be replaced by a

TABLE 6. Results of MTDT for the detection of *M. tuberculosis* in smear positive and smear negative specimens

Study (reference)	MTDT sensitivity (%) in different studies		
	Overall	Smear and culture positive	Smear negative, culture positive
Abe et al. (1)	92	100	70
Bodmer et al. (12)	71	100	14[a]
Jonas et al. (92)	82	100	54[b]
Miller et al. (126)	91	94	63
Pfyffer et al. (154)	95	100	80[b]
Portaels et al. (156)	86[c]	89	85
	97[d]	97	100

[a] 80% of these were positive only in liquid medium.
[b] ≤100 CFU/ml in culture.
[c] Belgian population.
[d] African population.

different one if the latter is not at least equivalent to the former and at most has the same cost. At present, amplification methods for *M. tuberculosis* cannot replace the conventional diagnostic techniques, especially since strains should still be cultured for susceptibility testing. The decision of the U.S. Food and Drug Administration is equally justified: use of the rapid MTDT should be restricted to smear-positive samples from untreated patients with tuberculosis and used only in conjunction with traditional sputum examination. It should not be used for smear-negative sputum samples or for other specimens such as pleural or cerebrospinal fluid (53).

(vii) Amplification techniques for *M. tuberculosis* drug susceptibility tests. Because the molecular basis of rifampin resistance is known (97, 189, 190, 212), up to 97% of the rifampin-resistant strains can now also be identified by PCR (35, 48, 83, 211). There is one important limitation to this test: it does not measure the proportion of rifampin-resistant mutants among the isolated strain. Only when the proportion is higher than 1% is the corresponding disease resistant to rifampin therapy. Only further studies will determine how frequently isolates with a low proportion of rifampin-resistant mutants are detected by this technique. Since rifampin resistance develops mostly in isolates that are already isoniazid resistant, the recognition of rifampin resistance lends a high suspicion of multidrug resistance.

Cultures remain necessary to identify rifampin-resistant strains not detected by the PCR, to test for susceptibility to other drugs, and to allow other investigations such as restriction fragment length polymorphism for epidemiologic purposes.

Fungi

Fungal respiratory infections may be due to dimorphic fungi such as *Histoplasma* spp., *Blastomyces* spp., or *Coccidioides immitis*, and they occur sporadically in defined geographic areas. We are not aware of any effort to diagnose these infections by molecular diagnostic techniques.

A second group of fungal respiratory infections are caused by ubiquitous saprophytic fungi, occur 10 times more frequently in immunocompromised individuals (204) than in nonimmunocompromised persons, and are common among patients in intensive care units. *Candida albicans* and *Aspergillus* spp. are the most frequent etiologic agents (204), and mixed infections with bacteria and cytomegalovirus occur in a significant proportion of cases. To shorten the time required for diagnosis, amplification reactions have been developed. Amplification targets have been genes coding for specific proteins (30, 95, 161, 186), 18S rDNA (15, 81, 82, 121, 124, 144), the 26S intergenic spacer region (180), or mitochondrial DNA (127). The last two represent repeated sequences, and thus their use increases the test sensitivity. In their work, Bretagne et al. (15) constructed an internal control. In some studies, primers were directed at a limited number (161) or a wide range of species; in the latter case, this was followed by treatment with restriction enzymes to obtain group identifications (82, 121, 173).

Molecular diagnostic techniques have been applied on BAL specimens and protected brush specimens to shorten the time for diagnosis, and on blood (26, 81, 128, 155) and/or urine (161) specimens in an effort to obtain a diagnosis through less invasive procedures. Only a few preliminary tests on detecting *Aspergillus* spp. in urine specimens have been performed (161). *C. albicans* was detected in seeded blood specimens (18, 81, 128), in blood samples from experimentally infected animals (95, 200), and in human blood in one study (95). The sensitivity of the PCR for *C. albicans* was disappointing: 79% (95), 73% (26), and 46% (158). Two possible reasons for this lack of sensitivity have been mentioned: the difficulty in releasing DNA from *C. albicans* cells, a critical need when they are present in small numbers (162); and the small volume of the specimen used in the amplification reaction (158). PCR has been used more frequently for classification and identification of *Candida* spp. than for their detection (93).

Spreadbury et al. (180) obtained a low sensitivity (80%) and specificity (72%) for the detection of *Aspergillus fumigatus* in clinical specimens, while Bretagne et al. (15), investigating a series of 55 specimens, obtained 25% false-positive results, i.e., detection of amplicons specific for *Aspergillus* spp. in immunocompromised patients who did not develop aspergillosis during follow-up. The authors point out numerous possibilities for contamination by environmental fungi during the preparation and storage of the reagents and the collection, transport, and manipulation of the specimens. Furthermore, the unsolved problem in the investigation of respiratory specimens for yeasts and molds is to distinguish between colonization and infection (15, 124, 134, 186). This differentiation might be possible in the future if genes related to virulence or invasiveness could be identified. At present, molecular diagnostic techniques do not improve the diagnosis of fungal infection by classical procedures.

Pneumocystis carinii

Several studies have confirmed the greater sensitivity of PCR over immunofluorescence for the detection of *Pneumocystis carinii* (22, 42, 46, 101, 111, 142, 185). Although the specificity of the assays is usually high, in one study *P. carinii* was detected in the absence of clinical symptoms (46). This could mean that colonization by *P. carinii* may occur, if contamination of samples in this study can be excluded. The conclusion of Tamburini et al. (185) that *P. carinii* should be sought in BAL specimens by the classical immunofluorescence microscopic technique and that amplification methods should be used only in exceptional cases, when the classical method remains negative, seems reasonable. In the presence of a high clinical suspicion of disease, PCR may have some utility, since claims have been made concerning the detection of *P. carinii* in sputum and two-thirds of blood specimens from patients with a generalized infection (113).

CONCLUSION

The statement that molecular diagnostic techniques, particularly PCR, are able to detect and amplify specifically a single molecule in solution in an olympic-sized swimming pool is nice but also illustrates one of the main difficulties of the procedure: how to introduce the contents of the swimming pool, or the one molecule it contains, into a 2-ml amplification vial.

The main problems facing molecular diagnostic techniques are the false-positive and false-negative results. The former may be avoided by the use of the correct controls in optimal working circumstances, i.e., good laboratory practice (177). Furthermore, any new or unusual findings should be confirmed by an independent amplification reaction. Laboratories engaging in molecular diagnostic techniques should first attain a proficiency level that excludes contamination.

Only when this technical level is reached is it possible to tackle the next problem—the test sensitivity. Much work remains to be done on this aspect. The sensitivity of use of oropharyngeal swabs and nasopharyngeal aspirates for the recovery of pathogens should be compared.

The unknown nature of most inhibitors in clinical specimens

certainly does not facilitate the development of techniques to eliminate them. Efforts to increase the sensitivity of a test by increasing the sample volume in the reaction mixture may increase the interference by inhibitors in some tests but apparently not in others. The extent to which procedures intended to concentrate the amplification target also concentrate inhibitors is unknown, as is the amount of target nucleic acid that is lost during procedures intended to eliminate inhibitors. The latter quantity could be determined by the addition of specific positive internal controls. New applications of amplification reactions should not be introduced without inclusion of specific positive internal controls. An optimal sample preparation method should be simple and rapid, and its ability to concentrate the target and eliminate inhibitors should not be nullified by its being too elaborate and time-consuming.

Compared with classical methods, nucleic acid amplification techniques are definitely more sensitive for the detection of some respiratory disease agents, particularly rhinoviruses, coronaviruses, *B. pertussis*, *M. pneumoniae*, and *C. pneumoniae*. These techniques are indispensable, not only for epidemiological studies but, for the last two organisms, also for clinical diagnostic purposes. However, in view of the results obtained in studies of other organisms, in which the sensitivity of the molecular diagnostic techniques is suboptimal, it can be surmised that the results for these agents are impressive only because the classical methods are particularly insensitive.

The great enthusiasm aroused by molecular diagnostic techniques in the field of tuberculosis detection should be tempered by the knowledge that the expectations concerning their high sensitivity and specificity have not yet been fulfilled. These problems must be addressed before amplification techniques can replace the classical diagnostic techniques. The lack of sensitivity of PCR for *M. tuberculosis* could result from the use of very small sample volumes in the reactions and an irregular dispersion of the organisms in paucibacillary samples. These shortcomings suggest the need for improved sample preparation techniques or the performance of more than one test on each sample.

The introduction of amplification techniques into the clinical diagnostic laboratory is also affected by the staff and space available and, if the decision is made to introduce them, whether they will be added to or replace existing procedures.

In conclusion, laboratories can apply molecular diagnostic techniques only if they comply with stringent external quality control requirements. As far as respiratory disease agents are concerned, amplification procedures should be limited to those listed above for which traditional culture methods are very insensitive and, depending on the geographical location, *Coxiella burnetii* and *Chlamydia psittaci*. For *M. tuberculosis*, they may be useful in some cases when an urgent identification is required if used in conjunction with culture in liquid medium and automated growth monitoring and for the rapid detection of most rifampin-resistant, and hence multiresistant, *M. tuberculosis* isolates.

We think that molecular diagnostic techniques are currently at a stage analogous to that of the clinical bacteriological techniques in the 1960s, before they were improved by many studies and gradually became standardized over the next two decades.

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